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STUDIES IN ANIMAL AGGREGATIONS: THE RELATION BETWEEN MASS OF ANIMALS AND RESISTANCE TO COLLOIDAL SILVER.

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One of us has been interested for many years in studying the causes and effects, particularly the physiological effects, of close aggregation upon animals. Much of the literature dealing with this problem has been surveyed elsewhere (Allee, 1927). The work on which the present paper is based was stimulated by the repeated reports of Drzewina and Bohn (1921–1926) of which the general results can best be summarized in their own words (1926):

"Nous avons recherché l'intervention du facteur *masse* dans les réponses de divers organismes vis-à-vis de multiples agents nocifs du milieu extérieur. Dans une masse M d'eau, on introduit une masse *m* d'un être vivant (*m* étant égal ou inferieur à M/100), cette masse résiste à un agent nocif déterminé (substance chimique par exemple); mais une masse plus petite, *m*/10 ou *m*/100, ne résiste pas; tout se passe comme si la masse de matière vivante exerçait, vis-à-vis d'elle-même, un effet protecteur (auto-protection)."

Drzewina and Bohn worked much with colloids of the heavy metals, particularly with colloidal silver. Accordingly we have used the same reagent, made as follows, from directions furnished by Dr. Terry-McCoy of the Department of Chemistry of the University of Chicago: "Dissolve 4 gm. of commercial dextrine in

100 cc. of water and then 4 gm. of pure caustic soda. Dissolve 3 gm. of silver nitrate in 20 cc. of water and add to the dextrine-soda solution. The precipitate of silver oxide that is formed is gradually reduced by the dextrine, the color changing to reddish brown. After 20–30 minutes, add 100 cc. of 90 per cent. alcohol and stir. Allow the mixture to settle for 15–20 minutes and then pour off the liquid from the particles of silver at the bottom. Add about 200 cc. of water and the silver particles will generally disperse; however, if this is not the case shake or stir until an even suspension of silver results."

With dilutions from such stock suspensions, it was easy to demonstrate, as Drzewina and Bohn state, that within limits, the greater the mass of animals, the better the protection. Thus with *Planaria dorotocephala,* 12 sets of two each exposed at room temperature to 10 cc. of water containing 10 drops of a rather weak suspension of colloidal silver, showed the beginning of head disintegration in from 4.5 to 10 hours; while 10 similar lots each containing from 10 to 75 worms, all lasted over 36 hours in the same volume and concentration. A species of Cladocera and the isopod, *Asellus communis,* showed similar, but less marked group protection.

Similar tests in which the animals were exposed to the action of dilute suspensions of colloidal silver for considerable time and were then washed and transferred to water similar to that to which they were accustomed, yielded such results as are listed in Table I. With the worms, the results are summarized in terms of survival after exposure, with added information concerning the number of worms that were able to crawl either with or without stimulation with a camel's hair brush. Other data at hand show that the protective effect of the group was greater than is indicated by this summary, but the summarized account is sufficiently conclusive. With the brittle starfish, *Ophioderma,* tests were made concerning the righting ability of the animals after exposure to colloidal silver. In these tests, after exposure, the washed animals were transferred to separate finger bowls each containing 250 cc. of well-aërated sea water. It will be noted that the mean righting time of the isolated animals is given in minutes while that of the animals tested in groups of five is given in seconds.

TABLE I.

SHOWING THE RESULTS OF EXPOSURE TO SUSPENSIONS OF COLLOIDAL SILVER FOLLOWED BY TRANSFER TO WATER SIMILAR TO THAT TO WHICH THE ANIMALS WERE ACCUSTOMED.

All were run at room temperature.

Animal.	Cc. of Water.	Drops Colloidal Silver.	Number Animals.	Time Exposed Colloidal Silver.	Survive.	Moving.	Died.
Planaria maculata...	15	12	6(3)	2 hrs.	11	2	7
	15	12	1(75)	2 hrs.	72	66	3
	5	1	5(3)	26 hrs.	9	0	6
	5	1	1(25)	26 hrs.	25	23	0
Planaria dorotocephala.....	10	2	40(1)	7 hrs.	15	1	25
	10	2	3(25)	7 hrs.	75	63	0
Dendrocoelum lacteum.....	5	1	5(3)	26 hrs.	3	1	12
	5	1	1(24)	26 hrs.	18	18	6
					Righted	mean time	
Ophioderma brevispina.....	50	2	48(1)	14.5–16 hrs.	41	30 min.	0
	50	2	12(5)	14.5–16 hrs.	59	38 sec.	0

More cases with different concentrations could be given but the result is the same in all. This agrees entirely with the experience of Drzewina and Bohn, who used a wide range of animals; and with that of Bresslau, using different toxic reagents with ciliates.

A priori one would expect exactly such a result since in each case there is much less of the toxic substance present per individual composing the larger groups, than when 1–3 animals are placed in the same amount of the same concentration of toxic substance (Pieron, 1921). Yet the possible menace of crowding, so frequently given as the only result of confining many animals within a small space, may easily be demonstrated even here. With the *Ophioderma*, for example, if the glass fingerbowls were covered with glass plates during the exposure to colloidal silver, thus stopping free gaseous exchange, the grouped animals were in much worse condition than were their isolated fellows.

CAUSE OF GROUP IMMUNITY.

When one begins to inquire into the causes of the greater immunity of the grouped individuals, he immediately finds division

of opinion. Drzewina and Bohn summarize their conclusions repeatedly in some such words as the following (1921*d*): "Bref, si chez diverses espèces que nous avons examinées jusqu'ici (Paramécies, Colpodes, Stylonychies, Stentors, Hydres, Convoluta, Glossiphonies, têtrads de Grenouille) tout se passe comme s'il y avait émission rapide d'une substance ou de substances assurant une défense; chez *Polycelis,* c'est le contraire (against KCl): on assiste, non pas à une *auto-protection* mais à une *auto-destruction.*"

They do not believe that the greater resistance of the group is due to a more rapid using up of the toxic substance, and cite two types of experiments to support their contention (1921). In the first type they expose some hundred (centaine) of Convoluta in one suspension and two each in the accompanying dishes. Even if the former receive 10 drops of colloidal silver to 25 cc. of water and the isolated animals have only two drops for the same volume, they find that the large group survives after the others are disintegrated. This is a fairer test than if the same amount of colloidal silver were added in both cases, but still it must be remarked that the ratio of colloidal silver per individual is about 10 times greater in the case of the two individuals, when compared with the larger group.

We have made some exact tests to cover this point and present the following summary of our results:

Animals Used: Planaria dorotocephala, 15–25 mm. long, exposed to action of colloidal silver for 7 hrs., then washed and transferred to water to which they were accustomed and examined on the day following the exposure.

I. 40 planarians isolated into 10 cc. of water plus 2 drops colloidal silver.

	No.	%
Normal, crawling when stimulated	1	2.5
Less than half disintegrated	2	5.0
Half and less than two thirds disintegrated	4	10.0
Two thirds but not all disintegrated	8	20.0
Wholly disintegrated	25	62.5

II. 150 planarians in 6 lots each in 250 cc. water plus 50 drops colloidal silver.

Normal, crawling when stimulated	0	0
Less than half disintegrated	0	0
Half, but less than two thirds disintegrated	18	12
Two thirds, but not all disintegrated	52	35
Wholly disintegrated	80	53

III. 71 planarians in three lots each in 10 cc. of water plus two drops of colloidal silver.

Normal, crawling when stimulated	63	88.8
Normal, not crawling when stimulated	1	1.4
Head started to disintegrate	7	9.8
Head completely disintegrated	0	0.0

IV. 75 planarians in three lots each in 10 cc. of water plus 50 drops of colloidal silver.

All wholly disintegrated.

The tests reported show clearly that if a group of 25 Planaria are placed in the same volume and the same concentration of colloidal silver as are isolated individuals, that the former survive the experience in good condition while the majority of the latter succumb, and almost all the others are severely affected. Further, if similar planarians are placed at the same time in the same volume of water, to which has been added sufficient colloidal silver to make the concentration per individual equal to that given the isolated worms, the bunched animals all succumb to a treatment that left 37.5 per cent. of the isolated individuals alive, even if most of them were strongly affected. And finally, if we place the groups in proportionate volume and proportionate concentration of colloidal silver, their condition approaches that of the isolated individuals. We conclude from this evidence that the greater protection which the mass furnished when exposed to colloidal silver is due to the smaller amount of the toxic substance which each individual has to remove from suspension in order to lower the strength below the threshold of immediate toxicity. The mechanism for this removal will be discussed later.

Another type of experiment was run with *Ophioderma* to determine whether or not the protective effect of the bunch was due to the fact that the bunched animals were exposed to a smaller concentration of colloidal silver than were the isolated animals. Since the area of the surface would affect the aëration and so affect survival values, the larger numbers were placed in crystallization dishes with a diameter of 24 cm., while the smaller bunches and the isolated animals were in the usual finger bowls with a diameter of 10 cm. The essentials of the set-up were:

One bunch of ten animals with 25 cc. sea water and 1.5 drops of colloidal silver each, and with a surface exposure of 45 sq. cm. per animal.

One bunch of 5 animals with 5 cc. of sea water and 0.3 drops of colloidal silver each, and with a surface exposure of 15.6 sq. cm. per animal.

Five isolated animals with 25 cc. of sea water and 1.5 drops of colloidal silver each and with 78 sq. cm. surface exposure.

A second set was exactly similar, except that the animals were exposed to 2, 0.4, and 2 drops of colloidal silver per individual.

The exposure in a typical experiment lasted 15.5 hours, after which the animals were washed as usual and transferred to fresh sea water for observation for the following 24 hours. At intervals records of numbers righted after having been turned over, and other evidences of activity, such as spontaneous motion of tube feet, were recorded.

All of the animals bunched in the 10 cm. fingerbowls recovered sufficiently to right themselves within 24 hours, despite the fact that four of the ten were much corroded by the action of silver that had settled to the bottom of one of the fingerbowls. In both experiments, the isolated animals fared next best, although they made a much poorer showing than did the bunched individuals that were exposed to a much lower amount of colloidal silver per animal; only six of this set of ten righted. The bunched animals, exposed to the same volume and amount of colloidal silver as those isolated, ranked a poor third, which may be due to the decreased surface exposure, or to some of the better known ill effects of crowding.

These experiments indicate either that the decreased amount of colloidal silver or the reduced volume of sea water present per individual, or both, markedly favored the survival of the animals bunched in the small amount of water present in the fingerbowls. Drzewina and Bohn emphasize the latter factor, and accordingly the following experiments were run to test out this point.

Lots composed of four solitary *Ophioderma* and one bunch of five were exposed in fingerbowls to 25, 50, 100, and 250 cc. of sea water, each containing only two drops of the stock suspension of colloidal silver. After 15 hours they were washed and tested for activity as usual. Those in 250 cc. of sea water, where the colloidal silver suspension had a value of one drop to 125 cc., righted immediately whether they had been bunched or isolated.

Those in 100 cc., with the colloidal silver ratio of 1 drop to 50 cc. all righted; the bunched animals in from 15 to 30 seconds, mean 27 seconds; the isolated in from 15 seconds to 8 minutes, mean 161 seconds.

The bunched animals in 50 cc., with the colloidal silver ratio of one drop to 25 cc., all righted in from 10 to 220 seconds, mean 101 seconds. Two of the accompanying isolated animals righted, one in 95 seconds and the other after an hour and a half. The others did not right in the next two days.

The bunch in 25 cc., with one drop to 12.5 cc. all righted in from 3 hours, 38 minutes, to about six hours, with a mean of 4 hours, 41 minutes. One of the isolated individuals righted almost immediately but none of the others did so. Contrary to the experience of Drzewina and Bohn (1921*a*), the toxicity depends on the concentration of the colloidal silver, rather than on the volume to which the animals are exposed.

It is worth noting that the protective action of the bunch appears only in the more toxic suspensions, where apparently the toxic strength per individual was more nearly reached even with the bunched animals. Drzewina and Bohn apparently would interpret such an observation as meaning that the auto-protective substance is secreted more rapidly in more toxic conditions.

Reconditioned Solutions.

The second and more convincing experiment used by Drzewina and Bohn as evidence in favor of their view that the group protection is furnished by some sort of auto-protective secretion, rather than by exhaustion of the toxic substance, is as follows (1921*a*):

"Nous décantons la solution où depuis 24 heures séjournent une cinquantaine d'embryons (tadpoles of *Rana fusca*) et dont la teinte révèle la presence du colloïde; nous y ajoutons le même nombre de gouttes que la veille, 1 par exemple, et nous y plaçons deux embryons neufs du même âge. Ceux-ci survivent, alors que des individus témoins, placés dans une solution neuve à 1 goutte de collargol, succombent, comme c'est la règle pour les isolés. Il semble ainsi que, attaquées par le colloïde, les larves émettent,

rapidement, une substance (ou des substances) qui a pour effet de les protéger."

Wholly similar experiments were run with the brittle starfish, *Ophioderma,* to test for the presence of the postulated auto-protective secretion. Colloidal suspensions in which *Ophioderma* had been exposed were reconditioned by three hours aëration with room air in order that the new lot might not suffer from low oxygen tension, and were filled to the original volume with distilled water. The original suspension had been made with one drop of colloidal silver for each 25 cc., and the same amount was added to this reconditioned water. Tests showed that the aëration of freshly prepared suspensions for this length of time caused a color change but did not markedly affect the toxicity.

Three lots, each consisting of one group of five bunched animals and four isolated individuals, were placed severally in 50 cc. of such a reconditioned suspension, nine hours after the previous experiment had closed. These were run simultaneously with three similar lots in freshly prepared suspensions of two drops of colloidal silver in 50 cc. of sea water. All conditions were similar. The temperature was 18.5°. The results are summarized in Table II.

TABLE II.

SHOWING RIGHTING TIME OF *Ophioderma* AFTER AN EXPOSURE OF 17 HOURS IN RECONDITIONED AND FRESH SUSPENSIONS OF COLLOIDAL SILVER.

All gave the righting reaction.

Reconditioned Suspension.

Bunch.			Isolated.		
Number.	Mean Time.	Spread.	Number.	Mean Time.	Spread.
15	73″	10″–12′	12	25″	5–118″
New Suspension.					
15	20″	7–75″	12	25″	10–65″

If Drzewina and Bohn were correct in thinking that the colloidal silver is not removed from solution on exposure to the animals, there should have been an excess accumulation of this substance in the twice used water; but after again conditioning

the used substance it was necessary to add the same amount, one drop of colloidal silver per 25 cc. to this suspension in order that it might be as deeply colored as was a fresh suspension made up with that amount of the colloidal silver. There was only sufficient colloidal silver left in this twice used suspension to discolor the liquid. Either the animals, or the aëration, or both, had removed the greater part of the silver, and it can be readily demonstrated that the effect was not wholly due to aëration. The water thus treated was distinctly sirupy, and evidently held organic matter, received from the two lots of *Ophioderma* which it had contained.

The effect of this twice reconditioned suspension was tested as before. Comparison tests were run with newly prepared suspensions, and the results are summarized in Table III.

TABLE III.

SHOWING RIGHTING TIME OF *Ophioderma* AFTER AN EXPOSURE OF 19 HOURS IN TWICE RECONDITIONED AND IN FRESH SUSPENSIONS.

Reconditioned Suspension.

Bunch.			Isolated.		
Number.	Mean Time.	Spread.	Number.	Mean Time.	Spread.
15	46″	10–221″	11 *	3° 27′	17″–22°
New Suspension.					
15	34″	10–175″	12	1° 30′	10″–14°

* One failed to give the righting reaction.

There is no evidence here of the presence of an auto-protective secretion in the sea water, protecting animals from the action of the colloidal silver, nor is there evidence for the presence of an active auto-destructive agent, which Drzewina and Bohn also postulated to explain certain cases in which the greater mass of animals exposed to KCl die more rapidly than do the solitary individuals. In the presence of an active auto-destructive secretion one would expect the mass to die more rapidly than do the isolated individuals, which is not the case.

There is good evidence that the secretion of slime or other organic matter into the suspension does remove the colloidal silver and so render the solution less toxic. It is particularly significant

that after having been twice used, the suspension required as much colloidal silver as was used in a fresh suspension to bring it to the same color. Similar results were obtained from other experiments, and additional evidence on this point will be presented shortly in connection with another aspect of the subject.

The work of Bresslau is of interest here. He finds that protozoa give off a substance which he calls " tektin," a mucin-like body. This is given off in greater abundance when the animals are stimulated by heat, pressure, methylene blue or iodin, etc. The " tektin," when given off, takes up water rapidly and exhibits strong surface activity, adsorbing foreign particles readily. Bresslau tells of putting 2 cc. of liquid from a culture of infusoria (either *Colpidium* or *Paramecium*) containing many individuals in one dish and a similar amount from the same culture, but with few individuals, into another. Into each he introduced one cc. of one per cent. solution of methylene blue. Both produced the " tektin " and adsorbed this poisonous material, that with the many animals so much more completely that the possibility of surviving the toxic action of the poisonous material was greatly increased.

Concerning the Specificity of Mass Protection against Colloidal Silver.

Drzewina and Bohn (1921*a*) in discussing the protection furnished by masses of tadpoles of *Rana fusca* exposed to the action of colloidal silver, as compared with isolated individuals similarly treated, say: " Nous cherchons à préciser la nature des substances protectrices, at à voir en particulier si elles sont specifiques," but they do not present data on this point.

There are two types of specificity possible. The protective secretion may have no other function and so be specific in that sense or it may be limited in protection to the species producing it. The first type of specificity will be discussed later. The latter aspect was investigated by placing one animal in a restricted volume of water containing a number of animals of a different species. All our observations show that the protective action of the mass is not limited to a given species. This is what would be expected if the fixing of the colloidal silver in some manner is the

principal element in the protective action. The results of typical experiments are summarized in Tables IV and V.

TABLE IV.

SHOWING THE NON-SPECIFICITY OF THE PROTECTION AGAINST COLLOIDAL SILVER.

Animals.	Water Cc.	Colloidal Silver Drops.	Time to Death. Hours.
Many *Cladocera*, one *Asellus*	12.5	8	Over 36
One *Asellus*	12.5	8	4.5
Many *Cladocera*, one *Planaria*	12.5	8	Over 36
One *Planaria*	12.5	8	5.5
50 *Asellus*, one *Planaria*	12.5	8	Over 36
One *Planaria*	12.5	8	5.5
Desiccated parotid gland, two *Planaria*	10	10	Over 36
Two *Planaria*	10	10	7.5
Snail slime, two *Planaria*	10	5	Over 36
Two *Planaria*	10	5	Less than 18
Snail slime, two *Planaria*	10	5	Over 36
Two *Planaria*	10	5	Less than 16
One *Physa*, two *Planaria*	10	5	Over 36
Two *Planaria*	10	5	11

TABLE V.

SHOWING COMMUNITY PROTECTION AGAINST THE ACTION OF COLLOIDAL SILVER.

Two drops of colloidal silver in 5 cc. pond water; exposure 13 hours, then washed and placed in pond water. Temperature 21°.

Animals.	Crawling.	Lived.	Died.
23 *Planaria maculata*	25	23	0
10 isolated *P. maculata*	0	1	9
25 *Dendrocœlum*, 1 *P. maculata*	1	1	0
30 *Glossophonia*, 1 *P. maculata*	0	1	0
26 *Asellus*, 1 *P. maculata*	1	1	0
25 *Segmentina*, 1 *P. maculata*	0	1	0
Pond moss, 1 *P. maculata*	1	1	0

As these tables show, such diverse organisms as *Cladocera*, *Asellus*, pond snails, pond leeches, *Dendrocœlum*, and even pond moss, if present in quantity, markedly protect planarians from the toxic action of colloidal silver. Even the actual presence of living organisms is unnecessary; snail slime without the snails protects efficiently apparently by adsorbing the colloidal silver. The slime becomes densely colored as the water becomes lighter in color. Suspensions in water of desiccated parotid glands of sheep exhibit similar adsorptive phenomena and have similar protective value.

A still more severe test of the specificity of the protection was made by placing recently killed *Asellus* in glass dishes containing 10 cc. of water plus two drops of colloidal silver, and introducing with these dead isopods one *Planaria dorotocephala*. At the end of 7 hours exposure, when 20 planarians isolated into a similar volume of the same concentration showed, 6, one third disintegrated; 13, one half disintegrated, and one wholly so, the five worms isolated into suspensions containing dead isopods showed one intact though bloated; 3, heads only, disintegrated; and one with both head and posterior end disintegrated. Thus the presence of the recently killed isopods decidedly protected the otherwise isolated worms from the toxic action of the colloidal silver. This, taken with all the other evidence at hand, furnishes convincing proof that the protective action of the mass against colloidal silver, extends beyond the species limits.

Discussion.

We have shown in the preceding pages that, within limits and other conditions being equal, there is greater protection the greater the mass of the animals present, when exposed to the same amount of colloidal silver in the same volume of water. Further, the protection is largely and perhaps completely, furnished by the fixation of the toxic substance by the mass of animals, so that each escapes receiving a lethal dose; while, with the same concentration, isolated individuals receive a stronger dose of the toxic substance. The colloidal silver may be differently fixed in different animals, but with those that secrete slime, like planarians, the colloidal silver is adsorbed on the slime. With other animals observed, it may be removed by adsorption on the surface of the animals themselves. Finally, as would be expected from this mechanism, we have demonstrated that the protection furnished by the mass, is, at least to a considerable extent, independent of the species present.

Our experiments do not support the favorite hypothesis of Drzewina and Bohn that group protection among these aquatic animals is furnished by the rapid production in the presence of a toxic agent, such as colloidal silver, of a more or less mysterious auto-protective secretion. It is true that the production of slime

by planarians actually serves as an auto-protective agent, not only in fixing colloidal silver in these experiments, but very probably in protecting the planarians from sudden changes in culture or habitat water. But slime production cannot be regarded as a specific auto-protective secretion, either in the sense that it is used for no other purposes, for obviously it plays many other roles in the economy of slime producing organisms, or in the sense that slime is limited in its protecting power to the one species producing it, since the protection furnished by aggregations of mixed species is easily demonstrated.

It is obvious that our experiments do not demonstrate that even in these cases there is no production of a more subtle sort of auto-protective secretion. Conclusions on this point must remain tentative pending the completion of appropriate quantitative chemical determinations. These experiments do make it unnecessary to assume the production of such a substance in order to explain group protection in all cases. Even the experiments with dead isopods are not conclusive on this point. In these experiments, the presence of freshly killed *Asellus,* in numbers, furnished marked protection for the solitary planarians. The supposition can be advanced that this protection was due to the diffusion of some chemical from the dead bodies. These experiments do demonstrate that the presence even of other living organisms is unnecessary for protection from the toxic effect of colloidal silver. So far as that is concerned, any other inert substance capable of fixing the colloidal silver by adsorption or otherwise should have the same effect.

This general phenomenon of the uptake and fixing of dyes and other possibly toxic reagents is well known, particularly by plant physiologists, who have developed a considerable literature concerning the method of fixation of such substances by living plants (cf. Scarth). The same phenomenon has been described for animals, and the protective value it furnished to a mass of animals has been recognized particularly by Bresslau.

In their work upon the relation between mass of animals present and toxic reagents, Drzewina and Bohn found that in the presence of certain chemicals, principally KCl, members of the group died more rapidly than did isolated animals. They explain such results

by postulating the production of an auto-destructive secretion. Their most impressive test for the presence of such an auto-destructive secretion was similar to that for the opposite effect, viz., the placing of a single individual into a solution in which a group of animals had died, and comparing the survival time of such an animal with one isolated into freshly prepared solution. We have no evidence to present on this point at present, but before accepting the hypothesis of the production of some specific auto-destructive secretion, we should need to examine the extent of the phenomenon, and the type of reagents that cause a marked increase in the destructiveness of the mass, particularly with regard to their effect on oxygen consumption and the elimination of waste products.

The deleterious effect of the crowding of many animals into close quarters has long been known and may be easily demonstrated. Goetsch has recently shown that different factors produce the unfavorable effect with different animals; thus, with *Hydra*, decreased food supply is most important; with *Planaria*, equally fed, the accumulation of waste products becomes the limiting factor; with tadpoles, the deleterious effect appears most closely correlated with frequent contacts between different members of the mass. Limitation of available oxygen has the same effect with all. Until it is demonstrated that chemicals such as KCl cannot produce the observed increase in the menace of the mass by their known effects upon one or all of these relations, it is idle to speculate concerning hypothetical auto-destructive secretions.

Our experience that protection is furnished by heterotypic aggregations as well as by those of the same species has important ecological implications. Hitherto biotic communities have been regarded as being organized by similar or dovetailing requirements which the organisms make of their environment. These may lead them to dwell in the same or in similar habitats, while animals with other requirements separate into a different community. Communities are also considered to be integrated by their food interrelations. (Forbes, Shelford.)

The work with colloidal silver, here reported, taken together with the observations of Bresslau and the experiences of the plant

physiologists, warrants the postulation of another and more subtle integrating factor in animal communities; and, when the observed effects of pond mosses are remembered, in biotic communities also. This integrating factor might well be called the auto-protective value of the community. Further investigations concerning the extent of the phenomenon in nature are needed, but the indications are that the secretion of such substances as slime, and probably even the accumulation in the surrounding environment of a certain concentration of what are ordinarily called waste products, help render a raw environment more suitable for many forms.

Drzewina and Bohn found tadpoles resisting colloidal silver more readily if the solution were made up in pond water rather than in tap water. They interpreted this as being due to the presence of some dilute auto-protective tadpole secretion, but from our experience it seems more probable that the protection was due to the presence of slime or other material secreted by all pond animals.

It is a commonplace of ecology that many species of plants and animals so react upon their environment as to limit the continued existence of their species in that locality, while at the same time preparing the way for their successors. This general condition is well illustrated by the work of Woodruff on the sequence of protozoan cultures. We are proposing an extension of this principle to include the idea that the pioneers in a sucession first react upon their environment so as to render it more favorable for supporting themselves and their own associates. It seems probable that a continuation of the same processes and further accumulation of the same products that at first are favorable will ultimately become unfavorable and so help cause ecological succession.

Conditioning of a raw environment to support a population is not limited to accumulation of favorable chemicals, for recent work (Allee, 1927*a*) indicates that in the absence of customary physical elements, as of eel grass in the case of *Ophioderma,* the animals may so react with each other as to substitute their own bodies for the missing grass.

It must be admitted that to date, most of the data supporting this view depends on laboratory studies made largely upon reagents

with which animals seldom or never come in contact in nature. Chemically clean glass dishes, filled with conductivity water given the proper osmotic value by the addition of chemically pure salts, and supplied with the optimal quantities of pure food do not furnish an optimum habitat for many animals, and it does not necessarily follow that the relations shown under such conditions would be duplicated in nature. There is, however, enough reason for thinking that similar relations may obtain in nature to justify further investigations.

Regardless of the protective mechanism, the fact that under certain conditions, masses of animals may have greater survival value for their components than would be the case if all were isolated, is of decided and general biological significance. In large bodies of water such aggregations might condition the water immediately around them, and in small bays where conditions become more pond-like, or in the more restricted area under small rocks, such conditioned water might have considerable permanence. In tide-pools, or in small streamside pools, the community auto-protection could easily have marked survival value. Even if it should appear that the only protection furnished is the distribution of the toxic agent among so many animals that none receive a lethal dose, the older conception that crowding is always a menace, hibernation and breeding season sometimes excepted, must be revised; and the indicated revision allows a better understanding of the persistence and probable significance of animal aggregations of a low level of integration.

REFERENCES.

Allee, W. C.

'26 Studies in Animal Aggregations: Causes and Effects of Bunching in Land Isopods. Jour. Exp. Zoöl., **45**: 255–277.

'27 Animal Aggregations. Quarterly Rev. Biol., **2**: 367–398. Bibliography.

'27*a* Studies in Animal Aggregations: Some Physiological Effects of Aggregation in the Brittle Starfish, *Ophioderma brevispina*. Jour. Exp. Zoöl., **48**: No. 2.

Bresslau, E.

'22 Die Ausscheidung entgiftender Schutzstoffe bei Ziliaten. Ber. Deutsch. Ver. Mikrobiol. Centrabl. f. Bakteriol. u. Parasit. I. Abt. Orig. **89**: 87–90.

'24 Die Ausscheidung von Schutzstoffen bei einzelligen Lebenwesen. Ber. d. Senckenberg. Naturf. Gesellsch. **54**: 49–66.

Drzewina, Anna et Georges Bohn.

'21 Variations de la susceptibilité aux agents nocifs avec le nombre des animaux traités. Compt. rend. Acad. d. Sci. **172**: 485–487.

'21*a* La défense des animaux groupés vis-a-vis des agents nocifs. *Ibid.*, **172**: 779–781.

'21*b* Action nocive de l'eau sur des stentors, en fonction de la masse du liquide. C. R. Soc. Biol. **84**: 917–920.

'21*c* Variations dans le temps de la resistance aux agents physiques et chimiques, chez *Rana fusca*. *Ibid.*, **84**: 963–965.

'21*d* Sur des phénoménes d'auto-protection et d'auto-destruction chez des animaux aquatiques. C. R. Acad. d. Sci. **173**: 107–109.

'22 Sur des phénoménes d'auto-destruction et d'auto-agglutination chez les *Convoluta*. *Ibid.*, **174**: 330–332.

'26 Influence de la température sur le sperme d'Oursin en fonction de la masse. C. R. Soc. Biol., **95**: 376–378.

Forbes, S. A.

'87 The Lake as a Microcosm. Peoria Acad. Sci., pp. 1–13.

Goetsch, W.

'24 Lebensraum und Korpergrosse. Biol. Zentrabl. **44**: 529–560.

Pieron, H.

'21 Note on the Work of Drzewina and Bohn. C. R. Soc. Biol., **84**: 919–920.

Scarth, G. W.

'26 The Mechanism of Accumulation of Dyes by Living Cells. Plant Physiol., 1: 215–231.

Shelford, V. E.

'13 Animal Communities of Temperate America. Univ. Chicago Press, 362 pp.

Woodruff, L. L.

'12 Observations on the Origin and Sequence of the Protozoan Fauna of Hay Infusions. Jour. Exp. Zoöl., **12**: 206–264.

'14 Effect of Excretion Products with Special Reference to the Protozoan Sequence in Infusions. *Ibid.*, **14**: 575–582.

POLYVITELLINY IN POND SNAILS.

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INTRODUCTION.

The occurrence of more than one vitellus, or embryo, in a single egg of some of the fresh-water pulmonates has never been satisfactorily explained. Although various forms of abnormal development which might be considered to have some relation to twinning, such as a *Campeloma* having two separate dextral shells (MacCurdy, '09), have been reported, such abnormalities were not observed in our laboratory stock or among the many wild individuals examined. The fact that a wild snail, number 5, Table I., was found to lay eggs having more than one vitellus when brought into the laboratory, suggested the idea that the laying of such abnormal eggs might be a transmissible character. When, however, in less than two months several of these abnormal, or polyvitelline, eggs had been laid by the controls and by other wild and laboratory reared snails, the genetic explanation seemed less likely. Fortunately, some of the stock from the projected

[1] Contribution from the Zoölogical Laboratory of the University of Pennsylvania and the Zoölogical Laboratory of the University of Michigan.

genetic experiment, the control and other isolated stock which was used in determining the viability of eggs laid by virgins (Crabb, '27*a*) furnish enough experimental data to show that the laying of polyvitelline eggs is not hereditary. Although the genetic experiment was discontinued, the occurrence of these abnormal eggs was noted in the records of other experiments.

Material and Methods.

Eggs of *Physa sayii, Lymnæa stagnalis appressa, L. columella* and *L. palustris* were used in this investigation. Egg masses of *L. s. appressa* and *L. palustris* containing polyvitelline eggs were placed in 5 per cent. formaldehyde for several hours, or even months; then the abnormal eggs were removed from the mass and mounted in hollowed slides, in 2.5 per cent. formaldehyde, to facilitate studying and drawing the vitelli. After a few weeks in the stronger solution the eggs ceased to shrink appreciably and began to darken but are still transparent after having been in the fluid eighteen months. The albumen of the eggs fixed in the strong solution over night and mounted in the weaker solution has neither coagulated nor blackened, and the vitelli are as plump and bright as they were when the eggs were sealed up over two years ago (Fgs. 3, 12).

In every instance the eggs recorded for individuals of each species represent all that that snail, or group of snails, laid during the period of observation. Thus, so far as the observations go, the data on the occurrence of polyvitelline eggs is much more reliable than if the masses had been collected at random.

Normal and Polyvitelline Eggs.

Normal eggs of the snails herein described consist essentially of an outer membrane enveloping a mass of albuminous substance which surrounds a relatively small, usually excentric, vitellus or yolk. Thus the structure of a pond snail's egg superficially resembles that of a bird.

Morphologically polyvitelline eggs of *L. s. appressa* and *L. palustris* differ from normal eggs only as regards the number of vitelli, and the amount of albumen displaced by the supernumerary vitelli. There is as much variation in size between normal eggs

(often of the same mass) as there is between normal and polyvitelline eggs. This is also true of the vitelli. Abnormal vitelli having the yolk protruding or flowing out, such as are shown in Figs. 3 and 11, and even cases of absence of vitelli, are occasionally found in eggs which to all other appearances are normal.

Several eggs have been found adhering together, but in most cases each one contained a single vitellus. In one instance (Fig. 7) two embryos are in a single egg while the other egg has none. In another case three eggs are joined together; the two end ones having no vitelli and the middle one having only two vitelli. The most remarkable instance of adhering eggs is one in which six normal eggs are joined in a string. However, there is nothing to indicate that polyvitelliny is dependent upon such adhesion of eggs.

The maturation and early cleavage stages are apparently normal in polyvitelline eggs having less than a dozen vitelli. In some eggs having a larger number many of the embryos pass the trochophore stage and some even reach the early shell stage before dying. The first maturation spindle is formed in a clear area or "well" in eggs which have been laid and moves to the periphery (Fig. 2) of the vitellus. The first polocyte migrates out into the albumen and the second remains attached, as has been shown is the case in normal eggs (Crabb, '27*a*).

We have never been able to hatch more than four snails from a single egg, but we have succeeded in repeating this experiment four times, and a fifth egg containing four embryos was killed and mounted. By tabulating the data available on the viability of polyvitelline eggs of *L. s. appressa* we find that of 53 eggs having two vitelli each, 39 hatched each vitellus, 8 hatched only one and 6 failed to hatch either vitellus. Of 5 eggs having three vitelli, 3 hatched all three, and 2 hatched only two of the vitelli. From 4 eggs containing four vitelli each, sixteen young were hatched. Of 116 *L. s. appressa* hatched from polyvitelline eggs not one was sinistral.

Occurrence of Polyvitelliny.

Twenty-one *Physa sayii*, fifteen of which were hatched from the same mass of eggs, deposited 348 masses containing 9,061 eggs, two of which had two vitelli each.

Of a large number of *Lymnæa columella* eggs taken at random only one contained more than one vitellus. This egg had two, one of which died in early cleavage and the other reached the gastrula stage.

Three F_1 and six F_2 of snail number 5, Table I., deposited a number of masses, which are not recorded in Table I., but none contained polyvitelline eggs.

Polyvitelline eggs occur most often in those masses which contain a large number of eggs (*i.e.*, 70 to 150 in *L. s. appressa*), and large masses are deposited by large, old individuals in the prime of life more often than small masses. This fact and the com-

TABLE I.

NORMAL AND POLYVITELLINE EGGS LAID BY TWENTY *Lymnæa stagnalis appressa*.

Individual snails designated $1F_{11}$–$1F_{16}$ are F_1 progeny of snail No. 1. Likewise, those designated $4F_{11}$, $5F_{11}$ and $5F_{12}$ are the F_1 progeny of snails No. 4 and No. 5. Snails Nos. 1–4 and 11 were adults when collected from lakes near Ann Arbor, Michigan, while all the others were reared in the laboratory. All the F_1 individuals and those numbered 6–10 inclusive were reared in isolation. The masses for each individual are consecutive.

Individuals.	No. Masses.	No. Eggs.	Masses Containing Polyvitelline Eggs.	Polyvitelline Eggs.	Vitelli in Each Egg.
1.....	70	5,453	13	23	2(20 eggs), 3, 4, 4
$1F_{11}$.....	13	844	6	14	2(8), 3(4), 5, 32
$1F_{12}$.....	7	560	2	2	3, 3
$1F_{13}$.....	6	364	1	1	3
$1F_{14}$.....	6	491	1	3	2, 2, 3
$1F_{15}$.....	6	443	0	0	0
$1F_{16}$.....	5	441	1	1	2
2.....	50	2,506	5	7	2(6), 3
3.....	1	105	1	25	2(22), 3, 4, 4
4.....	1	64	1	2	2, 2
$4F_{11}$.....	22	1,608	8	25	2(22), 3, 4, 4
5.....	7	466	5	19	2(16), 3, 3, *ca.* 26
$5F_{11}$.....	20	1,631	14	131	2(39), 3(12), 4(11), 5(3), 7, 9(3), 45, 6–15(29), 15–30(32).
$5F_{12}$.....	8	708	1	3	2, 2, 2
6.....	9	687	2	2	2, 2
7.....	8	497	0	0	0
8.....	4	246	0	0	0
9.....	20	935	0	0	0
10.....	7	487	0	0	0
11.....	14	1,327	6	15	2(15)
20.....	284	19,863	67	273	

TABLE II.

NORMAL AND POLYVITELLINE EGGS LAID BY ABOUT ELEVEN *Lymnæa palustris*.

The eggs in group 1 were laid by two snails kept in one aquarium; 2, eggs from an aquarium containing eight snails; 3, eggs laid by a single snail kept in isolation. The masses in each group are consecutive.

Group.	No. Masses.	No. Eggs.	Masses Containing Polyvitelline Eggs.	Polyvitelline Eggs.	Vitelli in Each Egg.
1.....	16	926	6	10	2(9), 3
2.....	91	4,824	1	1	2
3.....	21	1,189	2	4	2, 2, 3, 5
11.....	128	6,939	9	15	

paratively low number of eggs examined is probably the reason why polyvitelline eggs were not recorded for snails number 1 F_1 5, 7, 8 and 10, Table I. In the case of *L. palustris* the three individuals designated as groups 1 and 3, Table II., were large adults, but in group 2 only one was a large adult at the time we began examining the eggs. The data relative to the size and age of the twenty-one *Physa sayii* are not sufficient to determine whether or not the size of the individual and number of eggs in the mass are correlated with the presence or absence of polyvitelline eggs.

DISCUSSION.

The facts set forth in this paper indicate that polyvitelline eggs may be expected to occur among the normal eggs of old *L. s. appressa* in the prime of life, but that the laying of these abnormal eggs probably is not a transmissible character. The question whether or not the two or more individuals hatched from the same egg are true twins, triplets, quadruplets, etc., has been raised. The paper of Hall ('25) appears to be the only available record of "twinning" in Mollusca. He found eggs of the tubiculous mollusc *Serpuloides vermicularia* containing two embryos which he considered twins. Newman ('23) holds that true twins arise from a single cell and that twinning is "essentially a phenomenon involving a physiological isolation of equivalent parts of the blastoderm and a regulation of the isolated or twinned regions into complete embryos." He also states that he has "never seen a reference to a case of twins or double monstrosity in Mollusca

. . ." and attributes this to the fact that cleavage is determinate in animals of this phylum. From his explanation and his review of the works of writers who claim that twins, at least in the forms they studied, are produced by a process of budding (Patterson), fission (Stockard) or fusion (Gemmill) it is evident that the process of twinning begins later than the first cleavage of the egg. However, it might be possible for two ova to fuse before either has undergone cleavage; whether this would produce twins or not is another question.

We have been unable to find anything to indicate that the supernumary individuals developed from pond snails eggs arose from a single vitellus. Although some four dozen ovotestes of *L. s. appressa* were sectioned (Crabb, '27*a*) not more than ten and seldom as many as four vitelli were found in the ovotestis and hermaphrodite duct in any one snail and in no instance were ova, and rarely sperms, found in the filliform part of the hermaphrodite duct (*i.e.*, craniad to the region "O," Crabb, '27*b*, Fig. 1) which in life bends around the gizzard. Thus it appears probable that while actively contracting the gizzard inhibits the passage of ova to such an extent that several ova accumulate in the enlarged part of the hermaphrodite duct and during a period of reduced activity of the gizzard all pass into the convoluted uterus at one time. In this, or some other way, a number of vitelli become enveloped by the albumen and the egg membrane which would normally cover a single vitellus. Often a mass of sperms is enclosed with the vitelli (Figs. 1, 5). Thus, probably by sheer accident, a polyvitelline egg is produced, and for this reason the young hatched from such an egg should not be considered true twins any more than should all the other young hatched from that mass of eggs.

Summary.

That polyvitelline eggs do not contain true twins is shown by the following facts:

1. The vitelli are normally enveloped by the albumen and egg membrane before any of them have undergone the first maturation division (Figs. 2, 6).

2. The vitelli often occur in uneven numbers (Plate 1, Tables I., II.).

3. Motile embryos show no evidence of attachment to each other.

4. Each of the 116 young was a normal dextral individual, except for size, when hatched.

LITERATURE CITED.

Crabb, Edward D.

'27*a* The Fertilization Process in the Snail *Lymnæa stagnalis appressa* Say. BIOL. BULL., Vol. 53, No. 2.

'27*b* The Anatomy and Functions of the Reproductive System in the Snail *Lymnæa stagnalis appressa* Say. BIOL. BULL., Vol. 53, No. 2.

Hall, R. P.

'25 Twinning in a Mollusc, *Serpuloides vermicularia.* Science, 61, No. 1591, p. 658.

MacCurdy, H. M.

'09 A Double Snail. Rept. Mich. Acad. Sci., Vol. 11, p. 119.

Newman, Horatio H.

'23 The Physiology of Twinning. Chicago.

PLATE I.

Figs. 1–6 and 10–13 are of *Lymnæa stagnalis appressa* eggs; Figs. 7–9 are of *L. palustris* eggs. All figures except Fig. 2 were drawn with the aid of the camera lucida to the scale shown in the plate. The eggs represented by Figs. 3 and 12 were mounted in a very weak solution of formaldehyde in a slide having the pits so shallow that the covers flattened them somewhat.

Explanation of Figures of Polyvitelline Eggs.

1. An egg having four vitelli. The dotted line represents the extent of a mass of sperms in the albumen.

2. Five unreduced vitelli occurring in a single section through an egg. The first maturation spindle is shown in one ovum, and an aster of a first maturation spindle is shown in another.

3. An egg laid by a wild snail, showing seven vitelli. Yolk globules have been extruded from four of the vitelli.

4. An egg having seven vitelli in sixteen to thirty-two-cell stages.

5. An egg with three vitelli in early cleavage stages. The dotted line indicates the extent of a mass of sperms in the albumen.

6. An egg having seven vitelli which have not given off the first polar bodies.

7. *Lymnæa palustris* egg with two advanced embryos, one of which probably belongs in the small attached egg.

8. An egg having two vitelli, one of which is in the four-cell stage.

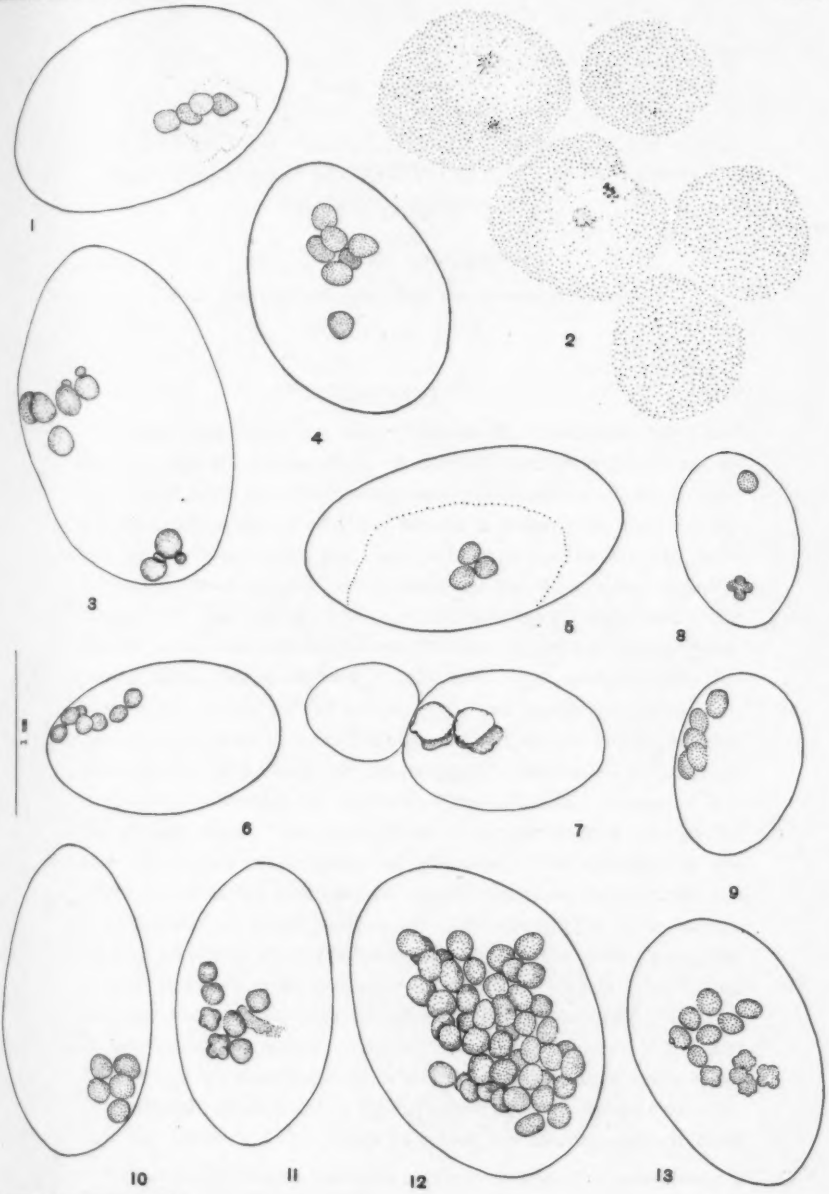
9. An egg having five vitelli in early cleavage stages.

10. *L. s. appressa* egg having five vitelli in early cleavage stages.

11. An egg having eight vitelli; one of which has disintegrated, while five are undergoing normal cleavage, and the other two apparently have not divided.

12. An egg having forty-six vitelli, laid by a wild snail.

13. An egg having twelve vitelli in four to eight-cell stages.



EDWARD D. AND RUBY M. CRABB.

THE EFFECT OF ALKALIES ON THE OXYGEN CONSUMPTION AND SUSCEPTIBILITY OF *PLANARIA DOROTOCEPHALA*.[1]

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I.

INTRODUCTION.

Considerable work has been done in this laboratory by Child and his students on the effect of chemical and physical agents on the modification of development and regeneration of organisms. For the interpretation of these results it is desirable that the action of the more commonly used chemicals on the rate of some metabolic process, such as respiration, be determined directly. Some work has already been accomplished along these lines, the effects of several substances on the rate of oxygen consumption having been tested: cyanides (Hyman, '19*a*), anesthetics (Buchanan, '22), caffein (Hinrichs, '24), and acids (Hyman, '25). The present paper is a contribution to this line of work and consists mainly of a study of the action of alkalies on the rate of oxygen consumption of *Planaria dorotocephala*. Hyman ('25) has already shown that acidification of natural water decreases the rate of oxygen consumption of *Planaria*. The question of the effect of increased alkalinity of natural water seemed of interest.

A number of investigations are available on the effect of increased alkalinity on various biological processes, such as activity, growth and development, and respiratory rate. Loeb ('98) found that the rate of embryonic development of *Arbacia* and *Fundulus* is accelerated in slightly alkaline solutions. Moore, Roaf, and Whitley ('05) also noted that bases in low concentrations favor growth and cell division in the fertilized eggs of *Echinus esculentus;* but Whitley ('05) failed to obtain any accelerating effect of

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alkaline solutions on the development of the teleost *Pleuronectes*. The fertilizability of *Arbacia* and *Asterias* eggs is increased by short exposures to alkaline sea-water according to Smith and Clowes ('24). Accelerations of activity by alkaline solutions have been observed by Dale ('13) in *Paramecium* and by Gray ('24) in the frontal cilia of *Mytilus edulis*.

Direct measurements of respiratory rate in alkaline solutions have yielded diverse results. In certain bacteria Brooks ('21, '22) noted the maximum rate of carbon dioxide production at or near neutrality with progressive decline in the rate with increasing alkalinity. The respiratory rate of the mold *Penicillium chrysogenum* is decreased 60 per cent. upon increasing the alkalinity of the medium from neutrality to pH 8.8 with NaOH (Gustafson, '20). According to Loeb and Wasteneys ('11) the rate of oxygen consumption of *Arbacia* eggs is increased 20–30 per cent. by raising the alkalinity of the sea-water with NaOH to pH 10.0 and increased 100 per cent. by raising it from pH 10.0 to 10.9. Similar results were obtained when ammonia was used. Thunberg ('09) found a decrease in the carbon dioxide production of excised frog muscle in solutions of NaOH, $Ca(OH)_2$, and $Mg(OH)_2$. Waldbott ('24) noted a slight acceleration of the respiratory metabolism of humans after the ingestion of alkaline solutions. The carbon dioxide production of tadpoles is increased in solutions of potassium and sodium hydroxide, but the rate of regeneration of the tail is retarded (Jewell, '20); Jewell regarded the increased respiratory metabolism as of a destructive nature.

From this review of the literature it is evident that both retardation and acceleration of biological processes result from exposure of organisms to alkaline solutions. In general, in the case of animal materials, an acceleration of growth and development and of respiratory metabolism in alkaline solutions has been found in the majority of cases.

Oxygen Consumption Experiments.

1. *Methods.*—The experiments consisted in determining the rate of oxygen consumption of planarians first in normal water

and then in water made alkaline to the desired degree by the addition of sodium or ammonium hydroxide. The general method of procedure was that described by Hyman ('19*a*). The species used was *Planaria dorotocephala*. Each experimental lot consisted of about 150 worms, 18–20 mm. long; these were selected from the laboratory stock and placed in a 500 cc. Erlenmeyer flask, in which they remained for a considerable time, being used in a number of experiments. They were fed in these flasks at intervals. At least three or four days were allowed to elapse after each feeding before the worms were used for experiment in order to avoid the increased respiratory rate consequent upon feeding (Hyman, '19*b*). The worms were fed sufficiently often to avoid any effects of starvation. From time to time new lots of worms were selected.

The water used was well water, an analysis of which is given by Hyman ('25). The alkaline water was prepared by adding enough sodium or ammonium hydroxide from stock solutions to raise this water to the desired alkalinity. The pH was determined with phenol red and thymol blue indicators by comparison with standard sets. At alkalinities greater than pH 8.6 a precipitate of calcium carbonate formed in the water. While there was no indication that this precipitate in any way affected the results, it was thought best to avoid it. This was done either by allowing the precipitate to settle and then decanting or by using carbonate-free water. This was prepared by adding 2 cc. concentrated HCl to eight liters of well-water and bubbling air through the water for 24 hours or more. That the rate of oxygen consumption of *Planaria dorotocephala* is the same in carbonate-free as in untreated well-water had previously been determined by Hyman ('25) and was verified in the present experiments.

Throughout each respiratory test, the flasks containing the worms and the blanks were kept in a large water bath at a temperature of 20° C. ± 0.5°. This was covered during the test. This darkening of the flasks together with the fact that the worms were kept continuously in the flasks was sufficient to eliminate movement. The worms remained very quiet throughout the oxygen consumption tests and the results cannot be ascribed to any differences in motor activity.

Two types of experiments were carried out: short exposures of a few hours to alkaline solutions, and long exposures of a week or more. Three experiments, that is, three flasks of worms, were generally carried on simultaneously.

2. *Short Time Experiments.*—In these experiments the rate of oxygen consumption of the worms was tested for two successive hours in normal well-water, pH 7.6 to 7.8. The water was then made alkaline by adding NaOH in varying amounts, giving a pH of 8.0 to 9.2 by intervals of 0.2 pH, or by adding NH_4OH similarly to alkalinities varying from pH 8.0 to 8.8. The rate of oxygen consumption was then tested in this alkaline water during the first third, and generally also fifth hours of exposure to it, freshly made alkaline water being used for each determination. As stated above, carbonate-free water was employed for alkalinities greater than pH 8.6. Twenty-three experiments were performed with sodium hydroxide and twelve with ammonium hydroxide. Table I. gives a typical experiment with each pH used for each of the two alkalies. The average per cent. of increase is based on all of the figures obtained in the alkaline solution. Table II. presents a summary of all of the short time experiments showing also the minimum, maximum, and average change in respiratory rate. These tables show that the respiratory rate was sometimes decreased in alkaline solution but was more generally accelerated. The normal variation in respiratory rate, based on twenty experiments where the respiration was determined in normal water for two successive hours, was 7 per cent. Only figures showing more than 7 per cent. alteration of the respiratory rate are therefore significant. From Table II. it can be seen that the higher alkalinities give generally a significant acceleration of the rate of oxygen consumption.

The worms would not survive alkalinities greater than those given in the table. They would live indefinitely in water made alkaline by NaOH to pH 9.0, but only twenty-four hours at pH 9.2. They were immediately killed in NH_4OH at pH 9.0. These results indicate that the action of alkali cannot be attributed solely to the hydroxyl ion.

It should be stated that the oxygen consumption of *Planaria* is independent of the oxygen content of the water at all ordinary

oxygen concentrations (between 7.0 and 2.0 cc. per liter, at least). The oxygen concentrations employed in the experiments was such (5 to 7 cc. per liter) that there is no possibility that the reduction in oxygen content by the worms during the experiment could have the slightest effect upon the amount of oxygen consumed.

TABLE I.

RESULTS OF A TYPICAL EXPERIMENT, SHORT TIME, AT EACH pH USED.

Figures represent cc. of oxygen consumed per hour.

	NaOH						NH_4OH			
Normal respiration in water. pH 7.6–7.8.										
	0.21	0.24	0.21	0.22	0.26	0.26	0.29	0.26	0.26	0.31
Respiration in alkaline water.										
pH	8.0	8.4	8.6	8.8	9.0	9.2	8.0	8.4	8.6	8.8
1st hr.	0.23	0.26	0.33	0.26	0.27	0.33	0.29	0.28	0.29	0.39
3d hr.	0.25	0.30	0.24	0.28	0.33	0.34	0.30	0.26	0.26	0.37
5th hr.	—	—	—	—	0.30	0.30	—	0.30	0.28	0.39
Average per cent. of increase of each of above.										
	14	16	32	22	15	20	1.5	7.6	6	23

TABLE II.

AVERAGE PERCENTAGES OF INCREASE AND DECREASE BASED ON TOTAL OF ALL SHORT EXPERIMENTS DONE AT EACH pH.

Figures are ± per cent. from the normal.

	NaOH						NH_4OH			
pH	8.0	8.4	8.6	8.8	9.0	9.2	8.0	8.4	8.6	8.8
Total no. exp.	3	5	3	6	3	3	3	3	3	3
Increase and decrease in respiration rate from normal.										
Min.	−12	−12	+11	− 4	+ 3	− 7	−20	−18	−10	+19
Max.	+19	+28	+61	+86	+26	+58	+ 3	+15	+17	+32
Av. of all exp.	+ 6	+14	+32	+22	+17	+33	− 7	+ 3	+ 5	+25

The results of all of the short time experiments are given in graphic form in Fig. 1, made from the data given in Table II. The rate of oxygen consumption is given on the ordinate, normal respiration in untreated water being taken as 100, respiration in

alkaline water as per cent. decrease or increase from this. The alkalinity in terms of pH is given on the abscissa. The solid line represents the results with NaOH, the dashed line with NH_4OH. As already explained only differences greater or less than 7 per cent can be taken as significant. In the case of NaOH the experiments at pH 8.4 to 9.2 show on the average an increase above the normal variation, but there is apparently no relation between amount of acceleration and degree of alkalinity. The drop at pH 8.8 and 9.0 is probably not significant. In the case of ammonia the curve rises steadily with increasing alkalinity but probably only the increase at 8.8 is significant.

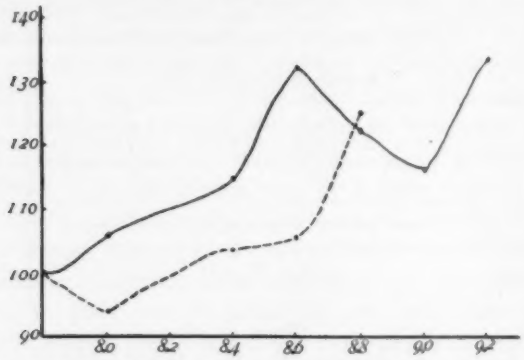

FIG. 1. Graph compiled from the average percentages of increase and decrease in respiration rate of all the short time experiments. See Table II. Per cent. of increase or decrease, normal respiration being taken as 100, on the ordinate and pH on the abscissa. Heavy line shows experiments in NaOH and broken line the experiments in NH_4OH.

3. *Long Time Experiments.*—These experiments consisted in determinations of the rate of oxygen consumption at frequent intervals in worms exposed continuously to alkaline water for periods of one to two weeks. Considerable difficulty was encountered in these experiments because the water would not remain at a definite alkalinity on account of the carbon dioxide given off by the animals. Various methods of keeping the water alkaline were suggested and were tried out. The carbonate-free water could not be used in the long time experiments, as, being unbuffered, it quickly became acid from the carbon dioxide given off by the

animals. Buffering this water with borax proved effective as a means of maintaining it at a definite alkalinity but the borax killed the worms in several days. Bubbling air through the flasks in the hope of removing the carbon dioxide emanating from the animals was not effective. When all of these methods failed, it became necessary to use the ordinary well water and to change it at frequent intervals. This proved fairly satisfactory. Boiling this water to remove dissolved carbon dioxide was of some help. The oxygen consumption of the worms was determined first in untreated well-water, pH 7.6 to 7.8. The water was then made alkaline to pH 9.0 with NaOH, decanted from the precipitate of $CaCO_3$ when necessary, and the worms kept in such water for one to two weeks continuously. The water was changed two or three times in 24 hours. Between changes, it became somewhat less alkaline but never fell below pH 8.6 throughout these long time experiments. The rate of oxygen consumption was determined every other day or every third day, always of course at pH 9.0. Owing to the fact that the worms lost weight during the experiment, because of starvation, for they were not fed throughout the period of experiment, it was necessary to weigh them in order to compare the rate of oxygen consumption at different intervals. The figures therefore represent the cc. of oxygen consumed per gram per hour. At the end of the experiment, the respiratory rate in normal water was again tested.

The results of three long time experiments which lasted one week are given in Table III., and of three more which lasted two weeks, in Table IV. In Table III., the oxygen consumption was determined first in normal water, then immediately in alkaline water (ph 9.0), then every other day in the alkaline water, and finally on the seventh day after the last test in alkaline water, again immediately in normal water. As shown in the table there was a marked acceleration of the respiratory rate during the latter part of the stay in alkaline water; this acceleration was immediately lost on return to untreated water, on the seventh day. The data in Table IV. give a similar result: acceleration during the exposure to alkaline water, immediate drop on return to untreated water.

TABLE III.

TABLE SHOWING RESULTS OF LONG TIME EXPERIMENTS IN BOILED WELL WATER, pH RAISED TO 9.0 WITH NaOH.

The pH never went below 8.6. Results expressed in cc. oxygen consumed per gram of animals per hour.

	1.	2.	3.	Av. % Increase 3 Exp.
I. day. Water pH 8.0	0.32	0.30	0.29	Normal. 100%
I. day. NaOH pH 9.0	0.28	0.28	0.30	− 5.5
III. day. NaOH pH 9.0	0.28	0.29	0.29	− 5.2
V. day. NaOH pH 9.0	0.44	0.26	0.41	+ 22.0
VII. day. NaOH pH 9.0	0.39	0.37	0.45	+ 33.0
VII. day. Water pH 8.0	0.24	0.26	0.25	− 17.0

TABLE IV.

TABLE SHOWING RESULTS OF LONG TIME EXPERIMENT IN ORDINARY WELL WATER DECANTED OFF PRECIPITATE.

The pH was raised to 9.0 with NaOH. The pH never went below 8.6. Results expressed in cc. oxygen consumed per gram of animals per hour.

	1.	2.	3.	Av. % Increase 3 Exp.
I. day. Water pH 8.0	0.21	0.18	0.19	Normal. 100%
I. day. NaOH pH 9.0	0.23	0.20	0.21	+ 10
III. day. NaOH pH 9.0	0.29	0.26	0.28	+ 43
VII. day. NaOH pH 9.0	0.24	0.23	0.24	+ 22
X. day. NaOH pH 9.0	0.24	0.23	0.21	+ 17
XIV. day. NaOH pH 9.0	0.27	0.29	0.23	+ 36
XIV. day. Water pH 8.0	0.23	0.20	0.18	+ 5

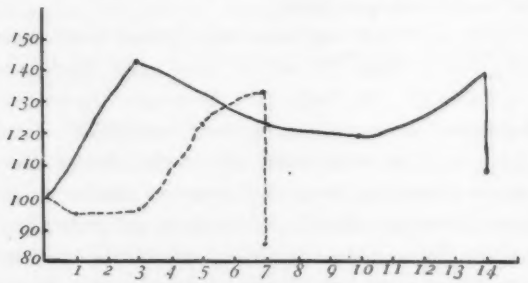

FIG. 2. Graph compiled from the data presented in Tables III. and IV. showing the results of long exposures of worms to NaOH solution, pH 9.0–8.6. Per cent. of increase or decrease, normal respiration being taken as 100, on the ordinate and number of days on the abscissa. Heavy line represents experiment in ordinary water and broken line experiment in boiled well water. The drop on the last day in each experiment shows the drop in respiration from NaOH solution to water.

The data given in Tables III. and IV. are graphed in Fig. 2, the solid line representing the data of Table IV., the dashed line, of Table III. The graph shows an acceleration of the rate of oxygen consumption during the greater part of the exposure to alkali, with an immediate drop at the end of each experiment on return to normal water.

Susceptibility Experiments.

The susceptibility method of determining differences in oxidative rate in different parts of the same organism and between different individuals of the same species was devised by Child and has been used extensively in this laboratory by him and his students. The method is explained by Child ('24). Briefly, the organisms to be compared are placed in toxic solutions of proper concentration or are exposed to lethal conditions and the various stages in disintegration are recorded. In general, the higher the oxidative rate, the shorter is the time before disintegration begins and the more rapidly does the disintegration progress. The method is therefore a rough means of comparing the relative rates of respiratory metabolism of comparable animals. Since the experiments on oxygen consumption had shown that the respiratory rate of *Planaria* is increased in alkaline solutions it seemed of interest to determine whether or not worms so accelerated would be more susceptible to chemical and physical agents than those respiring normally in ordinary water. A few experiments were tried to test out this point. Worms which had been kept for several hours in water made alkaline to pH 9.0 with NaOH were compared as to their susceptibility to toxic solutions and conditions while still in alkaline solution with control worms kept and tested in ordinary water or in solutions made up in ordinary water (pH about 8.0).

Comparison of susceptibility differences between different lots of worms is best accomplished by recording the progress of disintegration in certain arbitrarily selected stages. In the following experiments seven stages of disintegration were chosen: (1) worm entirely intact; (2) disintegration at the margins of the head; (3) from the end of marginal disintegration to complete disintegration of the head; (4) from the end of stage 3 to completion

of disintegration half way back to the mouth; (5) from the end of stage 4 to completion of disintegration to the level of the mouth; (6) from the end of stage 5 along the margins to the posterior end of the first zooid; (7) from the end of stage 6 to complete disintegration of the first zooid. The death of the secondary zooids is not considered. Ten animals are usually employed in each test, and the number of animals in each stage of disintegration is recorded hourly. The results are graphed by giving to each stage a numerical value, beginning with zero for stage 7, complete disintegration, and adding ten for each stage, intact animals being assigned the value of 60. The numerical value assigned to each stage is then multiplied by the number of animals in each stage at each observation. These values for the ten animals are then added together and divided by ten; the quotient thus gives the average stage of disintegration of the ten animals at the particular time of observation. Thus if at one observation, three animals were in stage 2, four in stage 3, and three in stage 4, their values would be 100,160, and 120, respectively, making a sum of 380; this divided by ten gives 38, or the average stage of disintegration reached, at the time in question.

1. *Experiments with Chemicals.*—To test the effect of alkalies on susceptibility to toxic chemical solutions, it was necessary of course to choose chemicals that were in themselves neutral. Anæsthetics were selected as the most convenient. Ethyl alcohol, 4 per cent., and chloretone, 0.1 per cent., were used. The anæsthetics for the control set of worms were made up in ordinary well water, those for the experimental set in the same water, made alkaline to pH 9.0 with NaOH. The results are shown in Figs. 3 and 4, being graphed according to the method explained above. Fig. 3 represents the results with alcohol, Fig. 4 with chloretone. In each graph, the solid line is the rate of disintegration of the control worms in ordinary water, the dashed line, the experimental worms in alkaline water. In each case, the disintegrating action of the anæsthetic is seen to be more rapid in alkaline solution.

2. *Experiments with Lack of Oxygen.*—When chemical agents are used the question of their penetrability is involved and complicates the interpretation of the results. For this reason it was thought advisable to kill the animals by other than chemical means

and determine the effect of alkalinity on the time of death. Lack of oxygen was one condition that seemed suitable for a test. A petri dish was divided into three compartments by two paraffin walls. A powder made by grinding up pyrogallic acid and sodium

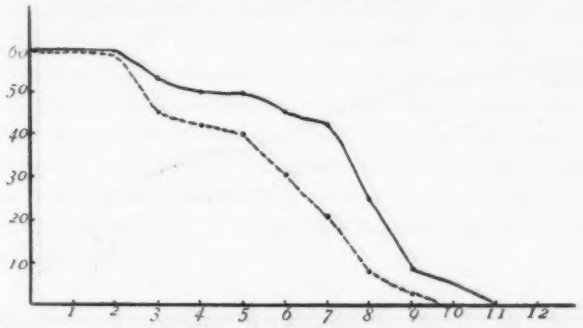

FIG. 3. Graph of disintegration gradient of worms exposed to NaOH solution and tested in alkaline alcohol (broken line) and worms not exposed to alkali and tested in neutral alcohol (heavy line). Alcohol used was 4 per cent. Hours on the abscissa and stages in disintegration on the ordinate. (See text for stages.)

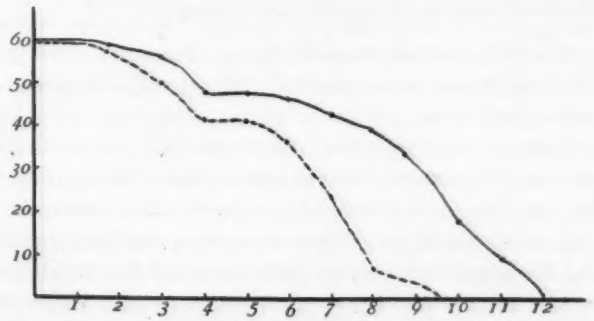

FIG. 4. Graph of disintegration gradient of worms exposed to NaOH solution and tested in alkaline chloretone (broken line) and worms not exposed to alkali and tested in neutral chloretone (heavy line). Chloretone used was 0.1 per cent. Hours on the abscissa and stages in disintegration on the ordinate. (See text for stages.)

hydroxide was placed in the center between the two paraffin walls. On one side of this were placed ten to twenty worms in ordinary water (pH 8.0); and on the opposite side in the third compartment, an equal number of worms of the same size and physiological

condition in alkaline water (pH 9.0). A cover was sealed on airtight. As the oxygen was absorbed by the alkaline pyrogallate, the worms began to die. The results of one such experiment are graphed in Fig. 5, the solid line representing the control worms,

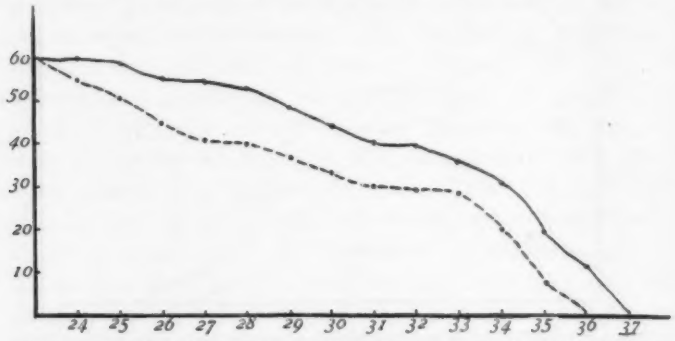

FIG. 5. Graph of disintegration gradient of worms exposed to NaOH solution and tested in alkaline solution in lack of oxygen experiment. Heavy line represents worms in ordinary water and broken line worms exposed to alkali. Record was not taken until disintegration began, *i.e.*, the twenty fourth hour. Hours are on the abscissa and stages of disintegration on the ordinate.

the dashed line, the experimental worms. In several experiments of this kind it was always found that the worms died faster from lack of oxygen at pH 9.0 than at pH 8.0.

3. *Experiments with Ultraviolet Radiation.*—Comparable lots of worms were exposed to ultraviolet radiation from a Cooper-Hewitt machine for a duration known to be lethal, one lot at pH 8.0, the other at pH 9.0. The worms were continually agitated during the exposure to insure uniform radiation. In all trials, the worms in the more alkaline water died more rapidly than those at the lower alkalinity. Death of course occurs only some hours after exposure to the radiation.

These experiments indicate that planarians are more susceptible to toxic chemicals and to lethal conditions when exposed to them in water of increased alkalinity, which is not in itself injurious.

4. *Susceptibility to Alkali.*—The question was raised whether the worms are able to acclimate to increased alkalinity. The long time experiments did not suggest that this was the case within two weeks' exposure, since the acceleration of the respiratory rate

endured through the period of the experiments. A further test by the susceptibility method was suggested. If any acclimation occurred, worms that had been kept in a non-injurious concentration of alkali should be less susceptible to a lethal concentration of alkali than control worms. Stocks of worms were kept in water made alkaline to pH 8.8 and 9.0, respectively, with NaOH, and were compared as to susceptibility to a higher concentration of NaOH with control worms living in ordinary water, pH 8.0. Tests were made every third day over a period of ten days. No difference in susceptibility between experimental and control worms was found. The resistance of *Planaria* to alkali was, therefore, not increased by continuous exposure, up to ten days, to alkaline water.

Discussion.

The experiments reported in this paper show that in general the rate of oxygen consumption is accelerated in water made more alkaline than normal, within physiological limits, by sodium or ammonium hydroxide. This increase in the respiratory rate lasts as long as the worms remain in the alkaline water, at least up to two weeks. Upon return to water of normal alkalinity, the respiratory rate drops at once. No evidence of any acclimation to the alkaline environment appeared in the course of the experiments. The oxygen consumption remained at a supernormal figure during continued exposure to the increased alkalinity. Susceptibility tests also showed no increased tolerance to alkali as a result of living for some time in water of increased alkalinity.

The question of the cause of the accelerated respiratory rate in alkaline water is of interest but the present experiments throw no light on the matter. It is rather generally accepted that alkalies increase permeability or have some other surface action (*e.g.*, Osterhaut, '14, and Warburg, '10). Such surface changes might well be the cause of the acceleration of the respiratory rate. It is not probable that penetration of the alkali into the interior is a factor in the acceleration for although ammonia penetrates cells readily it is believed that sodium hydroxide does not penetrate until the surface is actually injured. Since both alkalies caused an increase in the rate of oxygen consumption, the effect appears to be a surface one.

Not only is the oxygen consumption of *Planaria* increased with exposure to alkaline solutions but also the susceptibility to toxic agents and conditions is greater when such agents and conditions are applied in alkaline water. In the case of chemical agents, this increased susceptibility in alkaline solution might be ascribed to increased permeability. This explanation does not seem applicable, however, to the result with lack of oxygen and ultraviolet radiation. Exposure to these conditions is more rapidly lethal in water of increased alkalinity than in normal water. It seems necessary to conclude that the increased susceptibility of worms to chemicals and to toxic conditions when exposed in water of increased alkalinity is in some way associated with the accelerated metabolism of the animals in alkaline water. Susceptibility is thus again indicated as a method of measuring roughly differences in general metabolic rate.

Summary.

1. The general result of exposing *Planaria dorotocephala* to water whose alkalinity is increased from pH 7.6 or 7.8 to 8.0 to 9.2 by addition of NaOH or to 8.0 to 8.8 by addition of NH_4OH is an increase in the rate of oxygen consumption, whether the exposure is for long or short periods.

2. The increase lasts as long as the planarians remain in the alkaline water (longest experiment, two weeks). A return to the normal or to a lower rate (probably result of starvation) occurs at once when the animals are returned to water of the original pH.

3. The resistance of planarians to lethal concentrations of alkali is not altered by long exposure (ten days) to non-injurious concentrations of alkali.

4. The susceptibility of planarians to toxic chemical solutions, to lack of oxygen, and to ultraviolet radiation is greater when they are exposed to these conditions at pH 9.0 than when exposed at ordinary alkalinity of normal water (pH 7.8 to 8.0).

I desire to express my thanks to Professor C. M. Child and Dr. L. H. Hyman under whose direction the work was done.

BIBLIOGRAPHY.

Brooks, M. M.

'21 The Effect of Hydrogen Ion Concentration on the Production of Carbon Dioxide by *Bacillus butyricus* and *Bacillus subtilis*. Jour. of Gen. Physiol., Vol. 4, pp. 177–186.

'22 The Effect of Hydrogen Ion Concentration on the Production of Carbon Dioxide by the Tubercle Bacillus. Amer. Rev. of Tuber., Vol. 6, pp. 369–376.

Buchanan, J. W.

'22 The Control of Head Formation in *Planaria* by Means of Anesthetics. Jour. Exp. Zoöl., Vol. 36, No. 1.

Child, C. M.

'24 Physiological Foundations of Behavior.

Dale, D.

'13 On the Action of Electrolytes on *Paramecium*. Jour. of Physiol., Vol. 46, pp. 129–140.

Gray, J.

'24 The Mechanism of Ciliary Movement. The Relation of Ciliary Action to Oxygen Consumption. Proc. Roy. Soc. London, Vol. 96 B, pp. 95–115.

Gustafson, F. G.

'20 Comparative Studies on Respiration. XI. The Effect of Hydrogen Ion Concentration on the Respiration of *Penicillium chrysogenum*. Jour. of Gen. Physiol., Vol. 2, pp. 617–626.

Hinrichs, M. A.

'24 A Study of the Physiological Effects of Caffein upon *Planaria dorotocephala*. Jour. Exp. Zoöl., Vol. 40, No. 2.

Hyman, L. H.

'19*a* On the Action of Certain Substances on Oxygen Consumption. II. Action of Potassium Cyanide on *Planaria*. Amer. Jour. of Physiol., Vol. 48, No. 3.

'19*b* Physiological Studies on *Planaria*. I. Oxygen Consumption in Relation to Feeding and Starvation. Amer. Jour. of Physiol., Vol. 49, No. 3.

'25 On the Action of Certain Substances on Oxygen Consumption. VI. The Action of Acids. BIOL. BULL., Vol. 49, No. 4.

Jewell, M.

'20 The Effects of Hydrogen Ion Concentration and Oxygen Content of Water Upon Regeneration and Metabolism of Tadpoles. Jour. Exp. Zoöl., Vol. 30, pp. 461–507.

Loeb, J.

'98 Über den Einfluss von Alkalien und Säuren auf die embryonale Entwickelung und das Wachstum. Arch. f. Entw'mech., Vol. 7, pp. 631–641.

Loeb, J., and Wasteneys, H.

'11 Die Beeinflussung der Entwicklung und der Oxydationsvorgänge im Seeigelei (*Arbacia*) durch Basen. Biochem. Zeitsch., Vol. 37, pp. 410–423.

Moore, B., Roaf, H. E., and Whitley, E.

'05 On the Effects of Alkalies and Acids and of Alkaline and Acid Salts, upon Growth and Cell Division in the Fertilized Eggs of *Echinus esculentus*. Proc. Roy. Soc. London, Vol. 77B, pp. 102–136.

Osterhout, W. J. V.

'14 The Effect of Alkali on Permeability. Jour. of Biol. Chem., Vol. 19, pp. 335–343.

Smith, H. W., and Clowes, G. H. A.

'24 The Influence of Hydrogen Ion Concentration on Unfertilized *Arbacia*, *Asterias*, and *Chætopterus* Eggs. BIOL. BULL., Vol. 47, pp. 304–321.

Thunberg, T.

'09 Studien über die Beeinflussung des Gasaustausches des überlebenden Froschmuskels durch verschiedene Stoffe. Skand. Archiv. f. Physiol., Vol. 23, pp. 154–161.

Waldbott, G.

'24 Über den Einfluss von Säuren, Alkalien, und Neutralsalzen auf den respiratorischen Stoffwechsel des Menschen. Deut. Archiv. f. Klin Med., Vol. 143, pp. 325–333.

Whitley, E.

'05 A Note on the Effect of Acid, Alkali, and certain Indicators in Arresting or otherwise Influencing the Development of *Pleuronectes platessa* and *Echinus esculentus*. Proc. Roy. Soc. London, Vol. 77 B, pp. 137–149.

A CASE OF APPARENTLY ADAPTIVE ACCELERATION OF EMBRYONIC GROWTH-RATE IN BIRDS.

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Temperature experiments upon the egg of the domestic fowl have shown that the period of incubation varies inversely with the temperature at which the eggs are incubated. It may be assumed that this is due to an increase in the increment of embryonic development. If we consider any temperature well within the margins of safety for the egg (high 105° F. *ca.*, low 100° F. *ca.*) such as 102.5° F., we may say that an increase of one degree leads to an accelerated development while a decrease of one degree leads to a depressed or inhibited development. However, as far as I have been able to discover, no case is known in nature where an adaptive acceleration of the rate of embryonic development seems to have accompanied the evolution of new species within a group. The purpose of this paper is to report on what seems to be such an instance.

The cowbirds are a group of icterine birds comprising half a dozen species placed in three closely related genera:—*Agelaioides*, *Molothrus*, and *Tangavius*. Of these three, the first named is in every way the most primitive and doubtlessly represents the primitive stock from which the other two have evolved. The last mentioned is a side branch of the cowbird group and need not be considered in this paper. The species of *Molothrus* (and *Tangavius*) are parasitic in their breeding habits, *i.e.*, they do not make any nests of their own but deposit their eggs in nests of other birds and leave them to be incubated and hatched by these strange species. *Agelaioides* is not parasitic but is not entirely normal in its breeding habits. It breeds in old nests of other birds but cares for its own eggs and young. It will build for itself only if it cannot possibly get possession of a nest already built, showing that it still possesses some of its original nest-building instincts, but makes use of them only in case of emergency. In this genus the

female is extremely shy when on the nest and were it not for the protection of the male it is doubtful if in many cases the female would take sufficient care of the eggs. In *Agelaioides* the incubation period is 12½ to 13 days.

The genus *Molothrus* contains three species all of which are parasitic but have the parasitic habit developed to unequal degrees of perfection. The stages of perfection of the parasitic habit in these birds agree with what seems to be the phylogenetic relationships of the species. The most primitive member of the genus, *M. rufo-axillaris,* is parasitic on the still more primitive, non-parasitic *Agelaioides badius.* Its incubation period is the same as that of its victim and varies from 12½ to 13 days. The second species, *M. bonariensis,* is parasitic on a large number of small birds but has the parasitic habit very imperfectly developed, wasting large numbers of its eggs by laying in deserted nests, or even on the ground. The incubation period of this species is 11½ days. The third species of the genus, *M. ater,* has the parasitic habit best developed, lays its eggs in the nests of a great many species of birds, and does not waste its eggs as does *M. bonariensis.* This species has an incubation period of from 10 to 10½ days. (No bird in the world is known to have a shorter incubation period; few have one as short.) [1]

For the successful prosecution of a mode of reproduction such as parasitism implies it is obviously of great importance to the parasitic species to have a short incubation period as its egg may be laid in a nest after incubation has been started in the other eggs and yet must hatch as soon if not sooner than these others if the young parasite is to survive. (As a matter of fact in the majority of cases, irrespective of the species of bird victimized, the cowbirds' eggs hatch first.) Consequently when we find that with the perfection of the parasitic habit in the cowbirds (from the primitive non-parasitic *Agelaioides badius* to the relatively perfect *Molothrus ater*) there is a corresponding diminution of the period of incubation, or in other words, an acceleration of the embryonic growth-rate (amounting to about 20 per cent. difference between *A. badius* and *M. ater*) it is difficult to interpret the case as other

[1] The evidence on which the phylogenetic relationships of the species of cowbirds is based is contained in a manuscript now going the rounds of the publishers.

than adaptive. Shortness of incubation period could very easily have been of enough selective value for natural selection to operate on in a very decided fashion.

Lest it be thought that the differences in incubation period of the various species of cowbirds are due to differences in the extent of embryonic development, it should be noted that all of these birds hatch out in exactly the same stage of development. The size of the bird incubating the eggs seems to have no noticeable effect on the period of incubation. Birds as large as mockingbirds and as small as vireos hatch out young cowbirds in the same number of days. The size of the eggs of the various species of cowbirds is practically the same in all cases.

COMMENSAL ASSOCIATION OF A SPIDER CRAB AND A MEDUSA.

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During four successive years the writer was associated with a week-end biotal survey of the coast of South Carolina, and on two of these field studies encountered a curious commensal relationship between a spider crab and a medusa. The animals concerned were *Libinia dubia* Milne-Edwards and *Stomolophus meleagris* L. Agassiz, both of them common and typical inhabitants of the littoral zone of the Austroriparian Subregion.

Libinia dubia is a spider crab of moderate size with a rounded carapace averaging 6 cm. in diameter. It has long and slender walking legs and six median dorsal spines. Adult stages are confined entirely to the bottom zone where they crawl about in search of food. They are scavengers and members of the benthos.

Stomolophus meleagris is a rhizostome medusa of a diameter of 18 to 20 cm., hemispherical, without marginal tentacles, with eight rhopalia, and with fused oral lobes which form a thick cylinder, at the bottom of which are eight pairs of frilled lobes and a small central mouth opening. The margin of the umbrella is colored with a dark reddish-brown material, fading out aborally, which comes off freely and stains the hands when the specimens are picked up for inspection. They do not appear to possess any nettling organs, or at least none which could be employed defensively. This form is by far the most abundant Scyphozoan of the South Carolina coast, and is one of the more conspicuous planktonic organisms of the littoral zone.

Here we have to do then with a commensal relationship between a member of the benthos on the one hand and of vertically the most distant life zone, the plankton, on the other—certainly a most unusual arrangement. By means of this alliance the crab becomes a transient component of the plankton. The association

consists in the presence of the crustacean within the subumbrellar space of the medusa, clinging to the manubrium. Most of the crabs were observed with the head directed downward, but a few were situated with the head upward. In no case was more than one crab found in a single medusa. They resisted detachment, holding on tightly by means of the sharp and strongly curved ends of their legs, and would occasionally attempt to escape by scrambling around the manubrium, just as a squirrel does around a tree trunk. In such cases, however, their agility was not marked.

At Georgetown and at Murrell's Inlet, S. C., in the north-central region of the coastline, no examples of commensalism were observed. One *Libinia dubia* was taken on the rocks of the jetties at Georgetown, but no living medusæ were seen at either station. At Bluffton and at Sullivan's Island, in the south-central portion, the following records were obtained: Bluffton, three medusæ seined from relatively deep water, each containing a crab; no other examples of either species were seen. Between Sullivan's Island and the Isle of Palms, near Charleston, seventeen *Stomolophus* were taken by net and by hand from shallow water, and of these sixteen concealed a *Libinia*. In the remaining case, a crab was found on the bottom in the immediate vicinity of the jellyfish and the collector believed the crab to have just dropped from his conveyor, probably due to the commotion in the water which the numerous members of the party were causing. No other examples of either species were encountered, so this supposition is strongly probable. The commensal crabs varied in size but were all fully adult. At all four stations great numbers of stranded medusæ, mostly *Stomolophus* were seen along all beaches.

Thus it is seen that (1) along the South Carolina coastline both *Stomolophus* and *Libinia* are not uncommon and are frequently abundant; more so in the southern half, and especially true of the medusa as attested by the many cast-up specimens. (2) With a single exception, all examples of *Libinia dubia* captured were taken from the subumbrella of the medusa. (3) Again with a single exception, and that one a doubtful case, all examples of *Stomolophus meleagris* were found to harbor a crab. (4) All of the observations were made in the month of May and involved living adult stages of the commensals.

Several varieties of Hyperid Amphipods and also certain small fish are commonly known to seek shelter beneath the umbrella of various medusæ, but cases of large sized crabs occupying this situation seem to be rare. No references in the literature involving either of these types of animal as commensals were found by the writer, who is indebted to Dr. Mary J. Rathbun for the two following citations: Rathbun, '04, in writing of the distribution of Cancer jordani in Monterey Bay, California, notes "one under rocks between low tide mark and mean tide, and three from the subumbrella space of a large violet-and-white jellyfish" (species not stated). Weymouth, '10, writes: "*Cancer gracilis* is represented by a considerable number of specimens, both young and adult, all obtained by dredging, though in Puget Sound it is an abundant shore crab. On several occasions the young of this species has been found in considerable numbers clinging to the subumbrella of various medusæ. These have all been of small size—5 to 10 mm.—but it was not until the summer of 1908 that younger stages were found. On one occasion a number of medusæ were collected and in examining these many very young crabs were obtained and a smaller number of megalops. Some of these were kept alive until the molt to the young crab stage took place, so that there can be little doubt that the specimens were really the megalops of *gracilis*. Later megalops were obtained from other species of medusæ, and crabs of a slightly larger size than those found on the jellyfish were dredged in considerable numbers; it would appear, therefore, that in the case of *gracilis*, at least a considerable number of individuals pass that portion of their life history from the end of the free-swimming stage, probably early megalops, until reaching a size of 15 to 20 mm. clinging to medusæ, after which they drop to the bottom and live in the manner of the adult. I have found no other species than *gracilis* on medusæ though Miss Rathbun reports one specimen of *jordani* from the same situation. It would be interesting to know if this form of life history were universal with *gracilis* and if it were common in any other species." It will be noted that Weymouth is incorrect in the number of specimens observed as commensal by Dr. Rathbun, and also that no identifications of the medusæ involved are given.

The conclusion reached by Weymouth as to the cause underlying the association he reports is entirely plausible, but what is one to say in the case of *Stomolophus* and *Libinia?* It does not seem possible that either commensal was concerned with the food habits of the other; no crabs were observed feeding on dead specimens of the medusa, and though they may do so in deeper waters, this type of crustacean is not known to attack living prey. It is certain that the crab added the burden of freight to the jellyfish, but probably did it no other injury: also any possible benefit to the latter seems unlikely. On the other hand it is unquestionable that the crab received both shelter and transportation, though whether either or both of these benefits accounts for the condition is problematical. That the association has anything to do with the life history of the crab appears doubtful, since all specimens found were adults and long past the delicate stages of *Cancer gracilis* collected by Weymouth. The only other speculation which occurs to the writer is that the crab might resort to such shelter at the molting periods, but here again fact does not support theory, as those individuals observed all had fully hardened shells. The purpose of this curious union then remains to be determined by more extended and exact studies.

Another interesting angle of this situation is the problem of how the crab attains the medusa. Since the former is absolutely confined to the bottom in so far as its own efforts are concerned, there remain but two alternatives: either the medusa must descend to the substratum at least occasionally, and for an obscure purpose, or else one of the larval stages of the crab must seek shelter within the umbrella and then remain attached during a long period of its mature life, for a reason equally difficult to conjecture.

And if the facts of the case are not sufficiently strange, consider also the circumstance that this observation has so long escaped scientific detection and is also unknown to all of the local fishermen, boatmen, and sportsmen whom the writer interviewed, and this in spite of the familiarity of the animals concerned and the well nigh universality of the association in the region covered.

Spider crabs are famous for their protective adaptations, adorning the carapace with algæ, sponges, and hydroids, or possessing a shell so colored and sculptured as to blend effectively with the en-

vironment. Judging from this ever present condition, these crabs seem to require more than ordinary concealment from the numerous foes which prey upon them, and hence this factor may be the determining cause for their association with medusæ in the present case. In this connection it is of interest to point out a certain degree of mutual adaptation on the part of the associating species. *Stomolophus* has a spacious and deep subumbrellar area, within which its large guest finds snug but ample accommodation; the marginal aperture allows ready ingress and egress; the manubrium is deeply grooved and pitted, enabling the crab to easily cling to its steed with great tenacity; and nematocysts are absent or so poorly developed as to be ineffective. The *Libinia*, on its part, is admirably shaped so as to conform to the contours of this peculiar residence, the back being strongly convex and the abdominal surface slightly concave; the legs are curved inward and end in sharply pointed tips; and finally, the mid-dorsal spines of the carapace are well developed and would assist in maintaining the position, especially when the crab was situated in the upper regions of the subumbrella, the site from which all of the nineteen specimens here recorded were dislodged.

The writer wishes to acknowledge the assistance of Dr. Waldo L. Schmitt of the United States National Museum, who has kindly provided references and identification checks, by Dr. Bigelow for the medusa and Dr. Rathbun for the crab, at a time when original sources were not otherwise available. Specimens illustrating this association are deposited in the United States National Museum and in the zoölogical collections of the University of South Carolina.

LITERATURE CITED.

Rathbun, M. J.

'04 Decapod Crustaceans of the Northwest Coast of North America. Harriman Alaska Expedition, 10, p. 176.

Weymouth, F. W.

'10 Synopsis of the True Crabs (Brachyura) of Monterey Bay, California. Leland Stanford Junior Univ. Pub., Univ. Ser., No. 4, p. 42.

REGENERATION IN A TROPICAL EARTHWORM *PERIONYX EXCAVATUS* E. PERR.

G. E. GATES.

In the course of a recent study of the *Oligochæta* of Rangoon a number of earthworms were found with reproductive apertures in normal arrangement and position on the segment but displaced one to four metameres anterior to or one to three metameres posterior to their usual position. The prostates and ovaries had the same definite relations to the sexual orifices in such individuals as in normal specimens and hence were anterior or posterior to their ordinary position to an amount equivalent to the dislocation of the reproductive pores. None of the various metameric and organ anomalies that appear in nearly all of the species of Rangoon earthworms were noticed in specimens of the type just mentioned.

Morgan (1895) found similar anomalies in specimens of *Allolobophora fœtida* and suggested (p. 404) that "In those cases in which openings of the vasa deferentia occur on a segment anterior to the 15th metamere,[1] we may be dealing with a case of incomplete regeneration of the anterior metameres. . . ." But further on he says, "That all of the cases can be explained in this way is, I think, highly improbable," and (p. 450) "regeneration of the anterior end will not account for any of those cases where the vasa deferentia open on a matemere posterior to the fifteenth."

No references to hypermeric regeneration of anterior ends in earthworms could be found in the literature available. The abnormality (posterior dislocation) was found so frequently, however, that a series of operations was begun with the idea of learning whether or not such anomalous specimens would develop as the result of regenerative processes experimentally induced in earthworms of the species concerned. One of the animals first used seemed to have such an unusual capacity for replacing lost parts that an extensive series of experiments was initiated to discover the limits of this capacity. After several months it became

[1] In *A. fœtida* the normal position of the male pores is on segment fifteen.

necessary to discontinue the work. Some of the data accumulated are of such interest, however, as to warrant publication in a preliminary note. At the first opportunity the experiments will be resumed.

In the preliminary series of operations worms belonging to nearly all of the species occurring in Rangoon were anæsthetized and the anterior ends excised at various levels. Only individuals of two species, *Pontoscolex corethrurus*, and *Perionyx excavatus* survived more than five days after the operation. At the end of two months only a small amount of segmentally undifferentiated tissue had been produced at the cut ends of worms of the first species, whereas all operated specimens of the second species had regenerated, in much shorter time, segmentally differentiated anterior ends. Further experiments were confined to this second worm.

Adult specimens of *P. excavatus* attain a length of 130 mm., and a diameter of 5 mm. The prostomium is large, fleshy, and characteristic. The intersegmental furrows are deep and clearly marked. Secondary annulation is lacking. The dorsal and lateral parietes are heavily pigmented. The clitellum is ring-shaped, yellowish or gray, contrasting sharply in color with the non-clitellar segments, and lies between the intersegmental furrows 12/13 and 17/18. The setæ are numerous and arranged in a closed ring around each segment behind the first metamere. The spermathecal apertures are large paired pores in the intersegmental furrows 7/8 and 8/9 (paired spermathecæ in viii and ix). The single female pore is clearly visible on xiv (paired ovaries in xiii). The larger male apertures are closely approximated mid-ventrally on xviii (paired prostates in xviii, paired seminal vesicles in xi and xii, paired naked testes in x and xi).

Mature worms can be secured in large numbers all the year round. They easily adjust themselves to the conditions of life in the laboratory, and have an exceedingly low mortality rate after operation or injury. The sharply delimited clitellum and large sexual apertures enable rapid determination of position, while the clear cut intersegmental furrows, the absence of secondary annulation, and the distinctly projecting setal circles, render segment counting simple, and the detection of metameric and setal anoma-

lies easy. The dark red color of the normal segments sharply sets off the much lighter colored new tissue during the early weeks of regeneration and the presence of a protruding fleshy prostomium distinguishes a regenerating head from any other structure.

Only fully mature and normal worms were used. They were anæsthetized in a weak solution of chloretone and an excision was made by a clean cut, usually at an intersegmental furrow. The operated worms were kept in closed jars containing moist paper towelling. All results described in this paper were obtained in that part of the year known locally as the "cold season." The term is of course merely relative and indicates that the mercury is slightly lower all day long than at other seasons of the year. No effort was made to control the temperature but in the brick laboratory building the fluctuations of the mercury from midday to midnight are much less marked at this season of the year than out of doors.

In a short time after the operation a transparent conical outgrowth was visible at the cut end. In the case of regenerating anterior ends, oval fæcal pellets were found from the sixth day on, indicating that the digestive system had developed sufficiently in that short time to enable the worm to "bite" off, swallow, pass through the digestive system, and defecate pieces of the paper towelling. By the end of the second week the segmental differentiation of the regenerating heads was completed. Usually by the end of the third week the new segments contiguous to the old tail piece had attained the diameter of the metameres with which they were in contact, and were clearly setigerous. In the fourth week the pigment appeared in the regenerating segments.

Regeneration of Anterior Ends.

In all series of operations every surviving worm from which six or fewer segments had been amputated regenerated the exact number lost. If the prostomium or a fraction of the prostomium was removed a new prostomium or the missing fraction was replaced. In several worms a small wedge-shaped piece was removed dorsally, laterally, or ventrally from the anterior end of the stump. In each case such wedge-shaped pieces were replaced as well as the exact number of missing anterior segments. Only

one animal died following operation anterior to segment six and in this single instance there was reason for suspecting that the death of the worm was not caused by the effect of the operation on the individual but by another factor to be discussed later.

When the excision was made at an intersegmental furrow posterior to 6/7 but anterior to 18/19 the ratio of the number of segments removed to the number regenerated, varied and the percentage of failure to survive the operation was higher. Here again there was reason for suspecting that many of the mortalities following operation were due to something more than the effect of the operation itself. None of the amputations at intersegmental furrows 10/11 or 14/15 resulted in hypomeric regeneration, but the number of worms involved (five at the first furrow and two at the second level) is too small to permit generalization. At all other intersegmental furrows between 6/7 and 18/19 hypomeric anterior ends have been formed. The largest number of segments not replaced is four and the smallest is one.

In one series four worms regenerated hypomeric heads each having one "half segment," *i.e.*, a metamere extending across the whole diameter of the animal but antero-posteriorly only about half the width of the segments immediately in front of and behind it. Such half segments were not setigerous and may represent incompletely differentiated metameres, or perhaps growth zones, although the specimens concerned were killed in the fourth week after the operation, by which time the segmental differentiation is usually completed. In only one regenerating anterior end was a wedge-shaped half-segment found and this was ventral in position.

Excisions at levels 7/8, 9/10, 10/11, and 13/14 alone resulted in hypermeric regeneration but the total number of such cases is too small for generalization. Only one extra segment was formed in each case. In these hypermeric worms all the regenerated segments except of course the first were setigerous and clearly outlined by intersegmental furrows. No half-segments, wedge-shaped or otherwise were found in heads of this type.

When the anterior end was excised posterior to 17/18 only hypomeric heads of ten to sixteen segments were formed. The number of worms operated on behind this level was too small to

warrant a statement that total replacement of lost segments cannot take place posteriorly. It should be noted that when eighteen or more segments were amputated the digestive organs including part of the intestine which begins in the region of segment fifteen, as well as all of the reproductive organs were removed. When the cut was made between 17/18 and 24/25 twelve to sixteen segments usually regenerated. Posterior to 24/25 ten to fifteen segments regenerated. The posterior limit of anterior regeneration by tail pieces has not been determined, but it certainly lies unusually far back for earthworms, and at least in the last third of the length of the worm.

TABLE I.

SERIES TWO.

Anterior Ends from One to Twenty Segments Removed.

85 worms were amputated.
81 worms survived operation.
48 worms regenerated exact number of segments lost.
27 worms regenerated a smaller number.
5 worms regenerated a larger number.
1 worm failed to regenerate.

Table I. summarizes briefly the results obtained from a characteristic series of operations. Table II. summarizes the regeneration in the region lying between segments six and eighteen

TABLE II.

SERIES TWO.

Results of Operations between Intersegmental Furrows 5/6 and 17/18.

Number of Segments Removed.	Number of Specimens Operated.	Worms with Regeneration of Exact Number of Segments Lost.	Worms with Hypomeric Heads.	Worms with Hypermeric Heads.	Worms without Regeneration.
7	7	4	2	1	—
8	8	5	3	—	—
9	10	5	3	2	—
10	5	4	—	1	—
11	2	1	1	—	—
12	5	1	3	—	1
13	4	2	1	1	—
14	2	2	—	—	—
15	4	—	4	—	—
16	3	2	1	—	—
17	4	2	2	—	—

from the second series of operations. In Table III. is a condensed statement of hypomeric and hypermeric regeneration in the same region but including results of more than one series of operations.

TABLE III.

HYPOMERIC AND HYPERMERIC ANTERIOR ENDS REGENERATED AT CUTS BETWEEN SEGMENTS SIX AND EIGHTEEN.

Number of Segments Removed.	Number of Segments Regenerated.
7	3, 6, 8
8	6, 7
9	6, 8, 10
10	11
11	8, 10
12	8, 10
13	11, 14
14	— —
15	11, 12, 13
16	14
17	13, 14, 15

REGENERATION OF POSTERIOR ENDS.

No special attempt was made to study tail regeneration by anterior pieces. Numbers of amputated anterior portions were kept in conditions similar to those of the regenerating tail pieces and the daily records of the experiments contain some notes on these amputated pieces. Anterior ends of more than twenty segments may regenerate new tails. No information is available as to tail regeneration anterior to segment twenty. Amputation at various levels posterior to 20/21 usually resulted in rapid formation of new posterior ends. None of these regenerating anterior portions were kept alive longer than four weeks so that it is not possible to make any positive statement as to the ratio of segments replaced to those lost. There is no reason to suspect, however, that anterior portions of twenty segments or more cannot replace all segments especially if provided with food.

MUTILATIONS.

While the first series of experiments was under way a collection of worms containing several regenerating specimens of *P. excavatus* was brought into the laboratory. Arrangements were

at once made to secure several thousand worms of this species from various quarters of the town. A considerable number of individuals thus obtained had evidently been deprived in some manner of a head or tail or quite rarely of both ends. One collection of more than three hundred worms contained more than a hundred mutilated specimens. This percentage was so high that previous digging was suspected of being the cause of the mutilations. As the collections had been made on successive days over a period of several weeks, it is possible that some at least of the mutilated specimens were produced in this way. In order to avoid this factor, collections were made at several localities which presented every appearance of having been undisturbed for months. Such collections also contained high percentages of mutilated individuals. Practically all the mutilations found were amputations of a head or a tail at an intersegmental furrow. Only three regenerating individuals were found in which excision had occurred in the middle of a segment. In these specimens the missing half segment had been regenerated as well as a portion of the tail behind. One worm mentioned elsewhere had been deprived of dorsal portions of two segments in addition to the anterior end.

Table IV. summarizes the information available from records of the collections. It should be noted that all mutilations included within this table had been produced at least several days previous to the time of collection. Such few specimens as were brought into the laboratory obviously injured as the result of collecting processes were, of course, discarded and not included in the tables.

Through such collections more than fifteen worms were secured that had lost their heads. Nine were regenerating new anterior ends when brought into the laboratory. Of this latter number four were either immature or if mature had lost more than eighteen segments, for there were no characteristic sexual markings to make a determination possible. The remaining five specimens had lost their heads anterior to the prostatic segment. By assuming that the prostatic segment of each of these animals was the eighteenth metamere before the mutilation, as in normal worms, it was possible to determine the number of segments lost and the type of regeneration that ensued. On the basis of this assump-

tion three individuals had regenerated hypomeric anterior ends, one had produced a hypermeric anterior end and the others had exactly replaced the number of segments lost. The single worm with hypermeric regeneration had lost the first thirteen segments as well as dorsal portions of segments xiv and xv and had not only replaced the lost dorsal portions but had also formed *fifteen* perfectly normal and clearly outlined setigerous segments and the non-setigerous prostomial segment.

TABLE IV.

COLLECTION NUMBER 1.

Normal worms	231
Mutilated worms	104
Worms with tail mutilation	98
Worms with head mutilation	6
Worms regenerating a tail	77
Worms regenerating a head	3

COLLECTION NUMBER 2.

Normal worms	301
Mutilated worms	87
Worms with tail mutilation	84
Worms with head mutilation	3
Worms regenerating a tail	71
Worms regenerating a head	2

COLLECTION NUMBER 3.

Normal worms	1
Mutilated worms	6
Worms with tail mutilation	5
Worms with head mutilation	1
Worms regenerating a tail	3
Worms regenerating a head	1

COLLECTION NUMBER 4.

Normal worms	?
Mutilated worms	?
Worms regenerating a tail	49
Worms regenerating a head	4

It is evident therefore that both in its natural environment and under experimental conditions in the laboratory *P. excavatus* may regenerate hypermeric anterior ends. In view of this demonstration there seems to be no need for further search, at least for the present, for other explanation of the origin of the anomalous

specimens mentioned at the outset of this paper. Such abnormalities at least in the species under discussion appear to be adequately accounted for as the products of hypo- or hyper-meric regeneration.

VARIA.

Additional extensive experiments were begun to obtain information as to the posterior limit of anterior regeneration by tail portions, the anterior limit of posterior regeneration by head portions, the regeneration of pieces with two-cut ends from various regions of the body, etc. These experiments failed completely, as far as the objects in view were concerned, because of a constant series of accidents which will be described under the title of autotomy. A few notes from the records of these experiments are given herewith to indicate more clearly the unusual regenerative characteristics of this worm.

A. A short piece of eight segments from the middle portion of worm 109 was still living on the eighth day after the operation and responded quickly to various sorts of stimuli. Both ends had healed over without any signs of regeneration.

B. The forty-one anterior segments of worm 110 regenerated in nine days new tissue five and one half millimeters in length.[1] and fixing.

The new tail was composed of a long, metamerically differentiated portion containing more than thirty segments, the anal segment, and between these two a short region of formation of new metameres.

C. The anterior end of worm 132, a piece fifteen millimeters in length, composed of twenty-four segments regenerated in two weeks a tail fifteen millimeters long containing in the segmentally differentiated region, fifty-four segments.

D. A shorter anterior end from another worm regenerated at its posterior cut surface a *head* of several segments with a characteristic mouth and prostomium.

E. A twenty-three metamere fragment thirteen millimeters in length from the posterior half of worm 118 in two weeks regen-

[1] The measurements noted were made on material that had been killed by dropping into strong methylated spirits and then hardened in formalin. Old and new tissue appeared to be uniformly contracted by this mode of killing

erated at one end a new head containing thirteen segments and a prostomium and at the other end a tail two and one-half millimeters long, containing in addition to the anal segment and the growing region twelve differentiated segments.

F. A shorter portion from the posterior half of another worm regenerated at one end a head and at the other end a single anal segment.

G. A short tail fragment regenerated at its injured anterior end a structure exactly similar in appearance to the tail developed at the cut posterior surface of an anterior piece.

H. The nine anterior segments containing both pairs of spermathecæ were removed from worm 82. Eight segments regenerated. Characteristic spermathecal pores appeared in intersegmental furrows 6/7 and 8/9 (the posterior pair of pores being located between the last of the old and the first of the new segments!).

I. Several other worms from which anterior ends containing one or both pairs of spermathecæ had been removed regenerated heads with one or two pairs of spermathecal pores at various intersegmental levels. These worms were killed three weeks after the operation, hardened in formalin and dissected. Definitive spermathecæ had not been formed by that time. The site of each spermathecal pore was marked internally by the presence of a lump of soft spongy tissue. Some of these specimens with regenerated spermathecal pores were very similar to specimens of this species secured by Beddard (1886) from the Philippines. It is quite possible if not probable that many or even all of the thirteen anomalies described and figured by Beddard as "variations" were the result of regenerative processes.

J. Seventeen anterior segments were removed from worm 149. When the animal was killed at the end of the fourth week after the operation, seventeen segments had been regenerated. In the usual position on segment fourteen was a typical female pore. The clitellar segments (xiii–xvii) were distinctly lighter in color than the other new segments, indicating the beginning of clitellar differentiation. Although the head was carefully fixed and hardened the tissues were too soft and spongy internally for dissection and no reproductive organs could be demonstrated.

Autotomy.

Several references have already been made to disturbing factors which interfered with the success of some of the experiments. One of factors, the most important, was a tendency for the worms to break into fragments in early hours after the operation. For want of a better term this process of fragmentation will be referred to as autotomy. Only very rarely was this fragmentation observed to occur later than the first twenty four hours after the operation, and then only very small portions usually consisting of one or two segments were thrown off.

In the first series of operations on *P. excavatus* many of the operated worms autotomized one or more pieces from the posterior portion. In another series of anterior operations thirty two out of forty two animals autotomized portions of the tail ranging from seven to sixty millimeters in length. No series of operations in which tail portions of the worm were watched was free from this tendency to fragment. In the majority of cases one or two short pieces were autotomized from the posterior end of the major operated portion. Such fragments were usually dead when discovered but very often lacked the pungent odor so characteristic of decaying earthworm. Occasionally the fragmentation was much more striking and extensive. Worm *B* 9 from which eleven anterior segments had been removed broke into pieces. Worm *D* 19 from which *X* anterior segments had been removed broke entirely into pieces six to ten millimeters in length. Several other specimens from which eight or nine segments had been removed also autotomized extensively.

Autotomy is usually understood to be a throwing off by the animal of a small portion which usually dies without producing a new animal but in *P. excavatus* apparently any fragment from any region may survive, or more than one of the fragments may survive, with the survival determined by the presence or absence of something in the worm and not by the position of the fragment along the axis of the animal. The autotomy was observed only in posterior portions. The length of the tail however was of no significance. Posterior portions from one third to approximately nine-tenths of the length of the original worm autotomized

extensively while anterior portions longer than one-half never autotomized. Several posterior portions about equal to one-half the length of the original worm autotomized from each end one or two pieces which died while the longer middle portion survived.

The experiments were discontinued before a thorough analysis of various aspects of this interesting tendency to fragment could be completed. Two experiments, however, provided a hint, not only as to the cause of the fragmentation but also as to the cause of certain other disturbing factors. Worm 94 was anæsthetized as usual and cut into three approximately equal portions, each of which was kept separately in a tightly closed jar. No autotomy occurred in any of the jars. The head piece survived and regenerated a tail. The middle piece regenerated at one end an anal segment and at the other end a head about three millimeters in length composed of fifteen segments. On the day following the operation the tail portion was collapsed and flattened, dead, but without noticeable odor of decay. In the jar were three flies which must have been present in the tail portion of the worm at the time of the operation, as the jar was not opened until after the flies had appeared. Through the kindness of entomologists at the Imperial Bureau of Entomology, London, these flies have been identified as *Aphiochæta scalaris* Lw.

A number of head portions ranging from twenty-five segments to about half the length of the worm were kept in a single, large, tightly-closed jar. A few portions died during the first three days after the operations and were removed. At the end of the week four of the head pieces were still living and apparently in good health although without signs of regeneration. The only traces to be found of the other head portions were numerous tubular fragments of transparent cuticle. Crawling around inside the jar were numerous small insect larvæ. When the jar was opened two small flies very similar in appearance to those from the other worm escaped. There seems to be no reason for doubting that some larval stage of the fly was parasitic in these worms at the time of the operation and it is at least possible that the presence of parasitic fly larvæ is the factor responsible for the autotomy as well as other disturbances in the operated worms. It should be noted, however, that *A. scalaris* is a very general feeder and that

it has been bred from all sorts of decaying matter. The Director of the Imperial Bureau of Entomology writes: "I should be inclined to suppose that the attack on the earthworms that you have noticed was accidental, for it seems unlikely that this fly would prove to be a true parasite."

Summary.

1. *P. excavatus*, an earthworm occurring in large numbers in dung heaps and soil rich in decomposing organic matter in Rangoon has a regenerative capacity very much higher than any known at present from megadrilous Oligochæta with the single exception of the limnic *Criodrilus lacuum* Hoffm., from Europe. The rate at which regeneration is completed is rapid.

2. Posterior portions can replace the anterior segments lost if the number of metameres removed is seventeen or less. When more than seventeen segments are removed only ten to fifteen metameres were regenerated.

3. The posterior limit of head regeneration lies somewhere in the last third of the length of the worm.

4. Spermathecal apertures and female reproductive pores may develop on regenerating anterior ends.

5. Anterior pieces of twenty segments or more may regenerate tails.

6. A heteromorphic head may be regenerated at the posterior end of a very short anterior piece.

7. A heteromorphic tail may be regenerated at the anterior end of a very short tail piece.

8. A piece of twenty or more segments from the middle of the worm may regenerate at one end a tail and at the other end a head.

9. Regenerated heads may be normal, hypomeric, or hypermeric. Hypomeric and hypermeric regeneration is considered an adequate explanation of the origin of abnormalities described as anterior or posterior dislocation of the reproductive organs.

10. In collections made in various quarters of the town a high percentage of the individuals secured had been mutilated by the amputation of a head, a tail, or both. Many of the mutilated specimens were regenerating the lost parts when collected.

11. One or more pieces both anteriorly and posteriorly are very frequently autotomized after operation by posterior portions. Sometimes the whole tail portion fragments into pieces six to ten millimeters in length. Anterior ends have not been observed to autotomize.

12. A fly *A. scalaris* Lw. has been bred from portions of *P. excavatus*. Parasitism by this insect may possibly be responsible for the mutilated specimens and for the autotomy following operation.

REFERENCES.

Beddard, F. E.

'86 Description of Some New or Little-known Earthworms. P. Z. S., 1886, pp. 298–314.

Morgan, T. H.

'95 A Study of Metamerism. Q. J. M. S., Vol. 70, pp. 395–477.

STUDIES ON THE PHYSIOLOGICAL EFFECTS OF HYDROGEN CYANIDE.

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The question of the physiological effects of cyanide has been of considerable interest since Claude Bernard (1) in 1857 noticed that the venous blood of vertebrates is bright red after treatment with cyanide. It is well known that cyanides in dilute solutions act in general as protoplasmic depressants. In most cases this depressing effect can be attributed to the inhibition of oxidations. Investigations by Allen (2), Child (3), Hyman (4, 4*a*, 4*b*), Vernon (5), Buchanan (6) and others show that potassium cyanide, even in extremely dilute solutions, depresses reversibly the rate of respiration in *Planaria*. Lund (7), however, noted no decrease in oxygen consumption in *Paramecia* placed in potassium cyanide solutions. The fact that dilute solutions of cyanide act as anesthetics is equally well known (Heilbrunn, 8; Osterhout, 9). An important difference between the effects of a typical anesthetic, such as ether, and cyanide was pointed out by Heilbrunn (8) who showed that ether decreases the viscosity of protoplasm while KCN in anesthetic concentrations increases it. Heilbrunn, therefore, concluded that in the case of sea urchin eggs there are two types of anesthesia; in one the viscosity of the cytoplasm is decreased and in the other it is increased. The toxic action of cyanide in concentrated solutions is also well established (Hyman, 4). In vertebrates the toxicity of cyanide seems to be due to its effect on the central nervous system, as shown by Geppert (10) and Dontas (11). Child (12) showed that the portion of an organism with the highest rate of metabolism is most susceptible to cyanide, and that young organisms having a high rate of metabolism are more susceptible than adults with lower rates. For a detailed review of the literature on the various phases of the cyanide problem, the reader is referred to the paper of Hyman (4).

The question of the effect of cyanide on the permeability of membranes is a debatable one. It is generally considered that anesthetics, such as alcohol and ether, decrease permeability (Lillie, 13; Lullies, 14; McClenden, 15; Osterhout 9*a*). Wertheimer (16), on the other hand, concluded that narcotics increase the permeability of frog skin while Krehan (17) showed that KCN increases the permeability of plant cells to many substances.

Most workers with cyanide have used potassium cyanide, which in an aqueous solution is strongly alkaline, due to the manner in which it dissociates:

$$\begin{array}{c} KCN + H_2O \rightleftharpoons KOH + HCN \\ \Downarrow \\ K + \overline{O}H \end{array}$$

Hydrogen cyanide in an aqueous solution acts as an extremely weak acid, dissociating only to a slight degree. In view of the fact that the question of the effect of cyanide on permeability is a debatable one and since most of the previous workers have used KCN, it was thought advisable to study in detail the penetration of hydrogen cyanide through living membranes as well as its effect on the membrane.

The investigations were conducted at the Zoölogical Laboratory, University of Pennsylvania, for which privilege the writer wishes to acknowledge his indebtedness to Doctor C. E. McClung. The writer is also under obligations to Doctor J. H. Bodine, under whose direction the investigations were conducted, for many helpful suggestions throughout the progress of the work.

It was found convenient in this work to use the artificial "cell" devised by Jacobs (18) and constructed in the following manner: a hard glass tube 7 cm. long and 1.5 cm. in diameter was tapered at one end to an opening of one cm. in diameter and the tapered end provided with a tip. The skin from the hind legs or back of a freshly killed frog (*Rana catesbiana* or *R. pipiens*) was carefully stretched over the lipped end of the tube and held in place by a rubber band. The skin was so placed over the tube that the inside, or flesh side, of the skin was exposed to the exterior. The "cell" so constructed was placed in a 100 cc. quinine bottle and

both the "cell" and bottle fitted with rubber stoppers. The inside of the "cell" was filled with a borax-boric acid buffer solution and a solution of HCN in a borax buffer was placed in the quinine bottle. It was necessary to use a buffer which would not injure the skin or react chemically with either the cyanide or the silver nitrate solution used to determine the concentration of cyanide. The pH values from 6.8 to 9.2 were obtained by changing

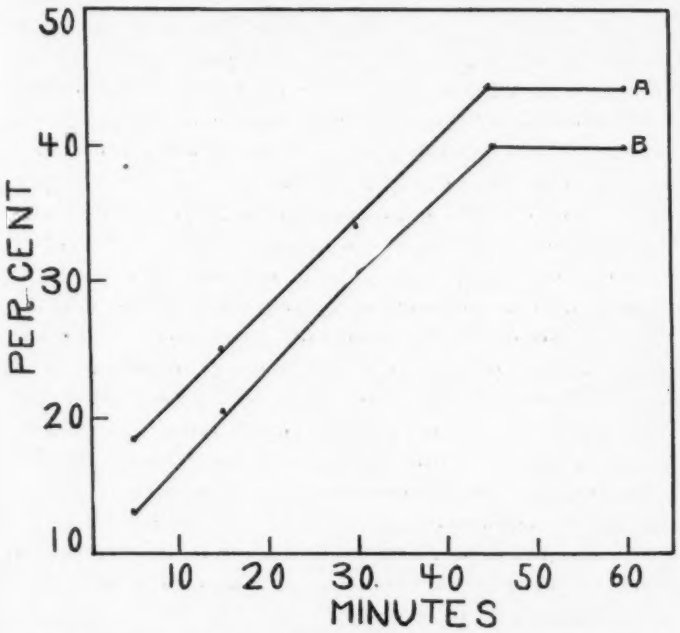

FIG. 1. Curve showing the relation of the position of the skin to the permeability of the skin to cyanide. *A*, skin with the flesh side out; *B*, skin normal, flesh side in. Abscissæ, time in minutes; ordinates per cent. cyanide.

the relative amounts of sodium borate and boric acid; for pH values below 6.8 it was necessary to add small amounts of nitric acid. The addition of HCN did not change the pH of the solution of either the borax or the borax plus nitric acid. Pure liquid hydrogen cyanide was used. No leakage occurred around the skin as shown by the fact that when a "cell" was filled with an

indicator and placed in a dilute solution of HCl the indicator did not change color. Several tests were conducted by reversing the skin and it was noted that the membrane was slightly more permeable to HCN when the skin was turned inside out than when it was in a normal position. The difference, however, was so slight that it is not significant (Fig. 1).

The "cells" after being filled with a borax buffer and put in a solution of HCN, were placed in a water bath at a constant temperature of 25° C. for one hour. At the end of that time equilibrium was reached between the cyanide inside and outside the cell. Five cubic centimeters of the internal and external solution were titrated with $N/50$ silver nitrate, using a one cc. pipette as a burette. The concentration of the cyanide solution used was $M/313$. That the skin was not killed at this concentration was easily demonstrated by substituting for the cyanide solution mineral acids, known not to penetrate living membranes. No change in intracellular acidity was noted in such control experiments. Experiments were conducted using external and internal solutions of various pH values, the external pH varied from 5. to 8.6 and the internal pH from 6.5 to 8.0. The results plotted in Fig. 2, show the relation of concentration of the total cyanide (HCN and CN) found in the cell at equilibrium to the various external and internal pH values. From this figure it may be noted that the penetration curve closely approximates the dissociation curve (x) and that the total concentration of cyanide inside the cell corresponds very closely to the undissociated cyanide in the external solution. The degree of dissociation represented in the curve was calculated from the formula

$$\log 1/H = \text{pH} = \log 1/K + \log a/1-a \quad (19),$$

where a = degree of ionization: K = dissociation constant, for HCN = 4.7×10^{-7} (20).

Figure 2 shows that at equilibrium, when the pH is from 5. to 5.5, the concentration of total cyanide within the cell is equal to the amount of cyanide in the external solution. From the dissociation curve it is evident that at this pH there is practically no dissociation, all of the cyanide being in the molecular condition. As the external pH was increased, the penetration curve rapidly

dropped. Likewise as the pH was increased the degree of ionization was increased. At a pH of 7.0 the dissociation is practically 50 per cent. and the concentration of cyanide in the cell is about 40 per cent. of the concentration of the external solution.

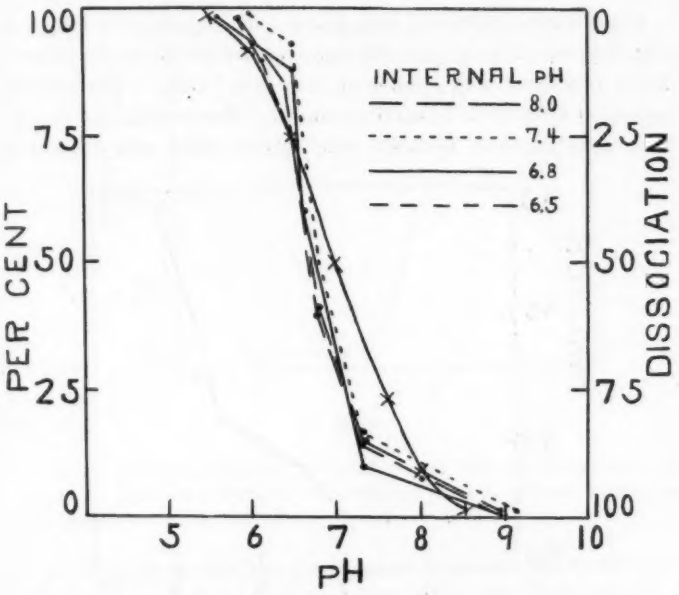

FIG. 2. Curve showing the effect of pH on the permeability of frog skin to HCN. Abscissæ represents the external pH; ordinates the per cent. of total cyanide on the right and the degree of ionization on the left. Calculated degree of dissociations represented by *X*. Internal pH values indicated as follows: short dashes 6.5, solid line 6.8, dotted line 7.4, long dashes 8.0.

At a pH of 9.0 dissociation is practically complete and accordingly little or no cyanide is present in the cell. It is evident that hydrogen cyanide seems to penetrate frog skin chiefly in the form of molecules and not as ions. It is also apparent from Fig. 2 that the intracellular pH varying from 6.5 to 8.0 does not affect the permeability of frog skin to hydrogen cyanide. Brooks (21) stated that changing the pH of the sap of Valonia with CO_2 and NH_3, changed the amount of 2 — 6 — dibromo phenol indophenol

that entered the cells. However, she states and also Scarth (22) shows that the pH of the sap in the vacuole could be no criterion of the pH of the protoplasm.

Experiments with Tadpoles, Daphnia and Elodea.

Experiments conducted with young bull frog tadpoles (Fig. 3) and *Daphnia* (Fig. 4) gave the same relative results as the experiments conducted with cyanide on frog skin "cells." After being separated from their cultural medium by cheese cloth, the organisms were placed in Syracuse watch glasses filled with a solution

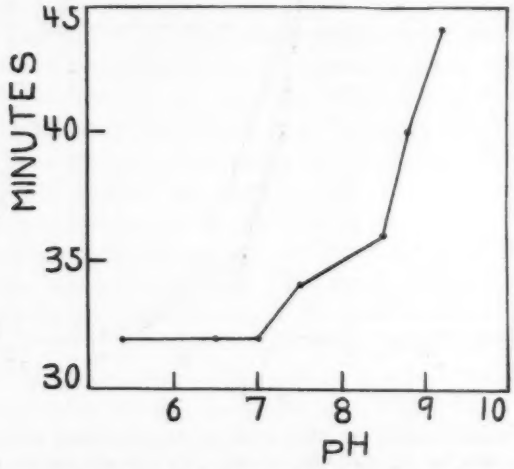

Fig. 3. Curve showing the effect of pH on the toxicity of HCN to young bullfrog tadpoles. Abscissæ represents the pH; ordinates the time in minutes required to produce death. Ten animals were used in each test.

of HCN in a borax buffer solution. It was previously determined that the animals were not killed in the buffer solution free of cyanide until after an exposure of three to four hours. On account of the anesthetic action of the cyanide, tadpoles did not prove to be good material for this work, since it was difficult to determine the time of death. When *Daphnia* was used the beat of the heart could be observed under a binocular microscope and the death point determined at the instant the heart stopped beating. It was noted, that the heart continued beating for some time after

other body movements had ceased and that the organism did not recover after the heart stopped.

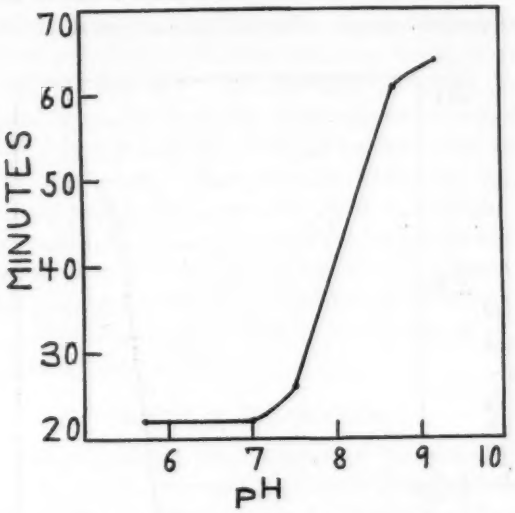

FIG. 4. Curve showing the effect of pH on the toxicity of HCN to *Daphnia*. Abscissæ represents pH; ordinates time in minutes required to kill 95 per cent. of the organisms.

It is obvious from Fig. 3 and 4 that less time was necessary to kill the animals in acid solution than in the alkaline solution. It required twenty-two minutes to kill *Daphnia* at a pH of 5.7 to 7.0; forty minutes at a pH of 8.0 and sixty-four minutes at a pH of 9.0. The concentration of cyanide used in the above experiment was $M/450$. The results indicated what would be expected from the study of the frog skin "cell," where more cyanide entered when the external pH was acid than when alkaline. The animals were killed first in solutions of the same pH values in which HCN penetrated the frog skin cells most quickly.

The effect of the pH on penetration of cyanide was further checked by studying its effect on the streaming of protoplasm in *Elodea* cells (in press). The streaming of the protoplasm can be observed under the high power of a microscope while the cells are immersed in a solution of cyanide. The pH was controlled,

as before, by a borax buffer. Leaves of *Elodea* near the growing tip of the branch were placed in a solution of hydrogen cyanide in Syracuse watch glasses and the time noted when the streaming of the protoplasm ceased. The results of a typical set of experi-

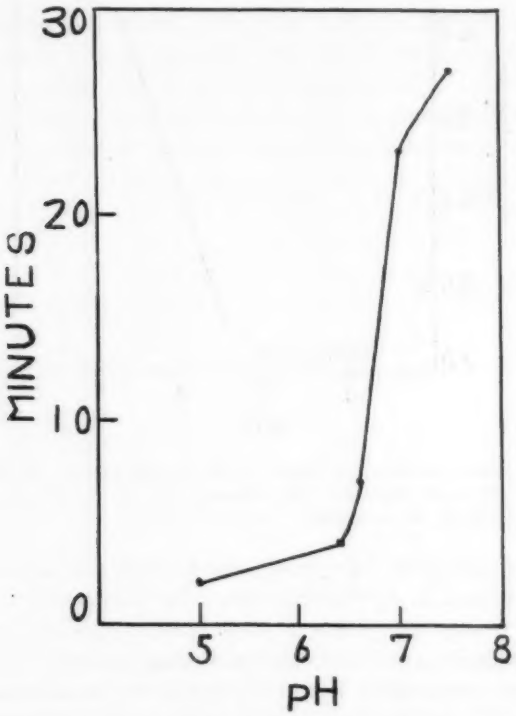

FIG. 5. Curve showing the effect of pH on the streaming of protoplasm in *Elodea* cells. Abscissæ represents pH; ordinates time in minutes required for streaming to stop.

ments are plotted in Fig. 5. Again the results show that the streaming of protoplasm stopped first in solutions of the same pH values in which HCN penetrated the artificial frog skin "cell" most quickly. Thus, in determining the toxicity of hydrogen cyanide to any organism, the value of controlling the hydrogen ion concentration of the solution is apparent.

It is obvious from the foregoing discussion that hydrogen cyanide penetrates living membranes chiefly in the form of molecules and in this respect is similar to other weak acids. Jacobs (18*a*) showed that carbonic acid killed various species of protozoa in a different order than mineral acids, which act primarily through their H ion (Collett, 23). This indicated to Jacobs, that the physiological effect of CO_2 was due to the entrance of the molecule. Beerman (24) and Bodine (25) obtained similar results with H_2S and HCN respectively. More recently, Osterhout (9*b*) and Osterhout and Dorcas (9*c*) showed by direct analysis that hydrogen sulfide and carbonic acid penetrated the living cells of *Valonia* chiefly in the form of molecules and not as ions. Brooks (21*a*) found that the amount of 2 — 6 — dibromo phenol indophenol in the sap of *Valonia* was proportional to the amount of undissociated dye in the external solution.

Effect of Temperature.

A series of experiments was conducted to determine the effect of temperature on the permeability of frog skin to hydrogen cyanide. The results are plotted in Fig. 6. It is evident from these curves that the higher the temperature the greater is the concentration of intracellular cyanide at any stated time. The curve suggests that of a typical unimolecular reaction as can be easily demonstrated by calculating the velocity constant from the following equation:

$K = 1/t \log a/a-x$ (26) in which x = the amount of cyanide in the cell at any time t; a = the amount of cyanide in the cell at equilibrium (Table I.).

Table I.

Velocity Constant K Calculated from the Unimolecular Equation $K = \frac{1}{t} \log \frac{a}{a-x}$ at Various Temperatures.

External and internal pH 6.8.

0°.				16°.				25°.				0°.				34.5°.			
t	*a*	*x*	*k*	*t*	*a*	*x*	*k*	*t*	*a*	*x*	*k*	*t*	*a*	*x*	*k*	*t*	*a*	*x*	*k*
Min.																			
15	1.84	.99	.0222	15	2.37	1.18	.0207	15	3.56	2.24	.0276	15	3.63	1.84	.0204	15	3.69	2.04	.0251
30		.158	.0256	30		2.17	.0357	30		3.11	.0252	30		2.97	.0256	30		2.90	.0223

It will be noted from Table I. that the velocity constant for any temperature is fairly consistent with the exception of 16°, and that K for the various temperatures is nearly constant. Plotting the log of the rate against the reciprocal of the absolute tempera-

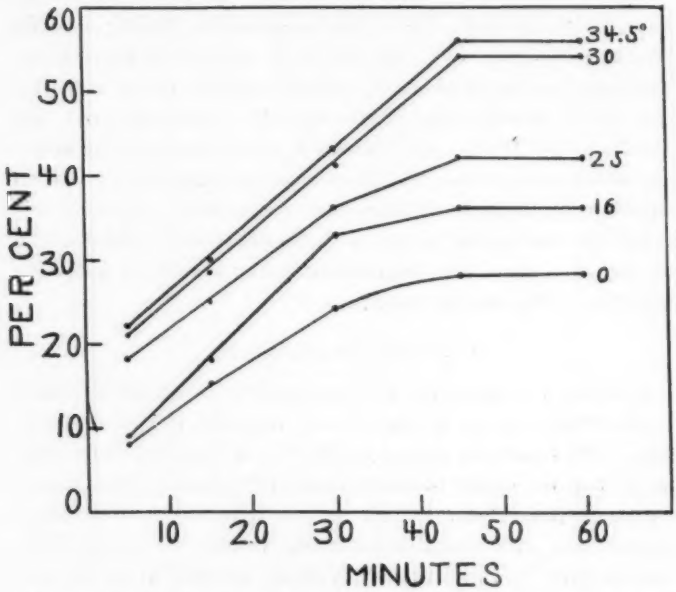

FIG. 6. Curve showing effect of temperature on the permeability of frog skin to HCN. Internal and external pH 6.8. Temperatures used were 0°, 16°, 25°, 30°, 34.5° C. Abscissæ represents exposure in minutes; ordinates per cent. of total cyanide.

ture, a curve was obtained as indicated in Fig. 7. The rate represents the time when the intracellular concentration of cyanide is twenty-five per cent. of the external concentration. It will be seen from this figure that there is a break in the line at 16° C. (.003415). Calculating Q from the Vant Hoff-Arhenius equation, (26)

$$K_2/K_1 = Q/2\left(\frac{T_2 - T_1}{T \cdot T_2}\right),$$

a value of 11,179 is obtained at a temperature from 16° C. to 34.5 and 4,300 for 0° to 16°.

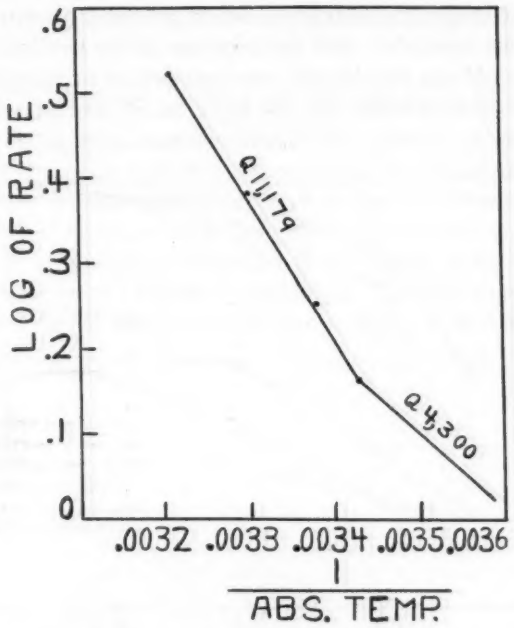

FIG. 7. Curve showing the log of the rate plotted against the reciprocal of the absolute temperature. Rate calculated from Fig. 5 as the time taken for the intracellular cyanide to equal 25 per cent. of the external concentration. Calculated from the formula

$$\frac{K_2}{K_1} = \frac{Q}{2}\left(\frac{t_2 - t_1}{t_2 \cdot t_1}\right).$$

Abscissæ represents reciprocal of the absolute temperature. Ordinates, the log of the rate.

RELATION OF CONCENTRATION.

Experiments were undertaken to determine the effect of the concentration of cyanide in the external solution on the penetration of hydrogen cyanide through frog skin. The external and internal pH and the temperature were maintained constant at 6.8 and 25° C. respectively. The concentrations of cyanide were *M*/109, *M*/124, *M*/160, *M*/196, *M*/225 and *M*/313. The results of such an experiment are plotted in Fig. 8. Each point on the

curve represents the average of three to five tests. It may be seen from this figure that, with the exception of the two low concentrations ($M/225$ and $M/313$), the total amount of cyanide within the cell at equilibrium was the same for all the concentrations. The rate of entrance of cyanide increased with an increase in

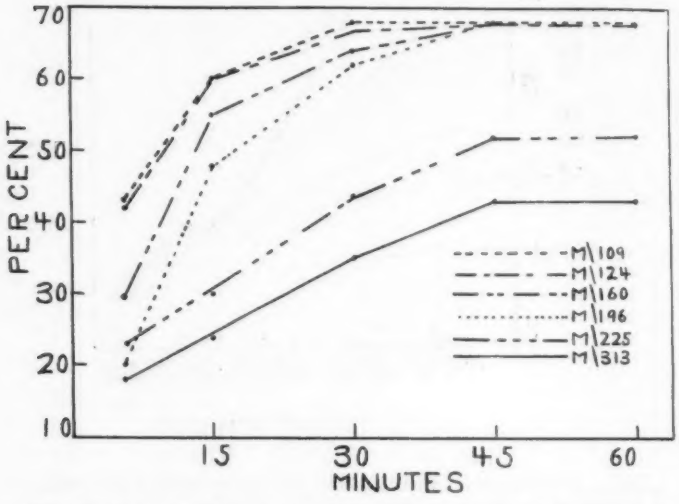

FIG. 8. Curve showing the effect of concentration of HCN upon the permeability of frog skin to cyanide. Concentrations of HCN used were $M/109$, $M/124$, $M/160$, $M/196$, $M/225$, and $M/313$. Abscissæ represents the time in minutes; ordinates per cent. cyanide.

concentration. At a concentration of $M/109$, equilibrium was reached within 30 minutes, while at lower concentrations equilibrium was not reached for a period of 45 minutes. The fact that the skin is not killed can be proved by substituting for the cyanide solution a mineral acid, which is known not to pass through living membranes, and testing the pH of the internal solution. As there is no change in the intracellular acidity, it is evident that no acid has passed through the skin.

Although, it is known that the frog skin is not killed by the above treatment with HCN, it is desirable to ascertain what effect the cyanide does have upon it. A series of experiments was conducted to determine the effect of hydrogen cyanide on the po-

tential difference of frog skin and the results are given below in detail.

Effect of Hydrogen Cyanide on the Potential Difference of Frog Skin.

Osterhout (9*c*) and others have shown that the electrical resistance of an organism is an excellent indicator of its vitality and that death is accompanied by an increase in permeability. An increase in permeability is equivalent to an increase in the electrical conductivity or to a decrease in resistance. In order to determine the physiological effect of hydrogen cyanide on frog skin, it was

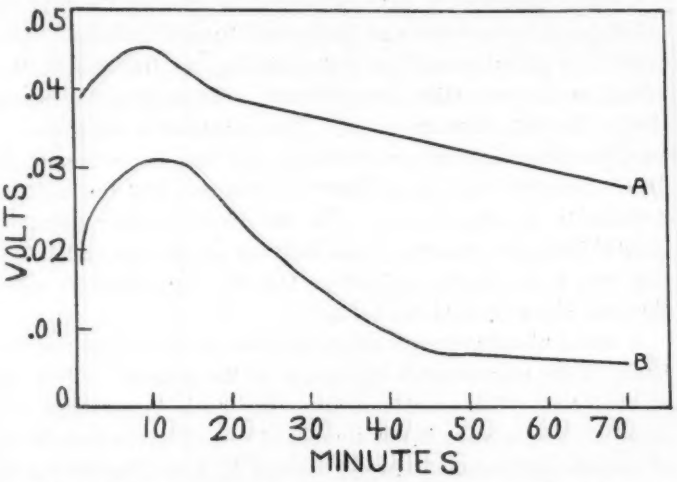

Fig. 9. Control experiments; curve showing the P.D. of frog skin in *A*, Ringer's solution and *B*, borax-boric acid buffer. Abscissæ represents the exposure in minutes. Ordinates P.D. in volts. Different pieces of skin from the same frog were used.

potentiometer. The same solution was placed inside and outside the cell. The skin from the hind legs of bull frogs (*Rana cates*- thought advisable to study its potential difference before and after being immersed in various solutions of this chemical. The apparatus was similar to that previously used in the first part of this paper with the exception that non-polarizable zinc electrodes were placed inside and outside the cell and the potential read on a

biana) was used. Since the potential difference of the skin from different frogs and also different pieces of skin from the same frog, varied considerably, it was necessary to repeat each experiment many times and only characteristic curves of single experiments will be given. There is some evidence at hand to indicate that the conditions under which the frogs are kept influences to some extent the potential difference of the skin. It was observed in several instances, when the temperature of the vivarium dropped several degrees below normal, that the potential difference of the skin also dropped and that on warm days the potential difference was usually higher than that at other times.

Control experiments were conducted by placing the skin in solutions of borax-boric acid buffer and Ringer's solution. The result of a typical control test is given in Fig. 9. Readings on the potentiometer were taken every minute. The temperature varied during the tests from 20–21° C. The experiments were run in parallel series using different pieces of skin from the same animal. It will be noted from Fig. 9, that there was an initial rise in P.D. followed by a gradual decline. The two curves are approximately parallel indicating that the borax buffer is no more toxic to frog skin than is the Ringer's solution. The pH of the Ringer's solution was about 8.2 and the borax 6.8.

A series of experiments was undertaken to determine the relation of the concentration of cyanide to the potential difference of frog skin. The cyanide solution was made by adding pure liquid HCN to a borax buffer at a pH of 6.8. The concentrations of cyanide used were $M/136$, $M/154$ and $M/225$. Fig. 10 shows the results of a typical set of experiments. The cells were placed in a borax buffer for ten minutes, then removed and placed in the cyanide solution. The skin was allowed to remain in the cyanide solution for various periods of time, then removed and placed in a borax buffer free from HCN. The period between arrows indicates the time that the cells were exposed to cyanide. In all cases it may be observed that the skin completely recovered after being removed from the cyanide solution. After the cells had been removed from the borax solution and placed in cyanide solution at a concentration of $M/225$, a great stimulation occurred, followed by a gradual drop in the potential difference to the base line. This

stimulation, characterized by a rise in potential difference, is also evident at a concentration of $M/154$ but not nearly to the same degree as with the weaker concentration of cyanide. The drop following stimulation at a concentration of $M/154$ is practically of the same magnitude as obtained with a $M/225$ solution. In the case of the $M/136$ solution there was no stimulation but the potential difference dropped suddenly to the base line. The skin

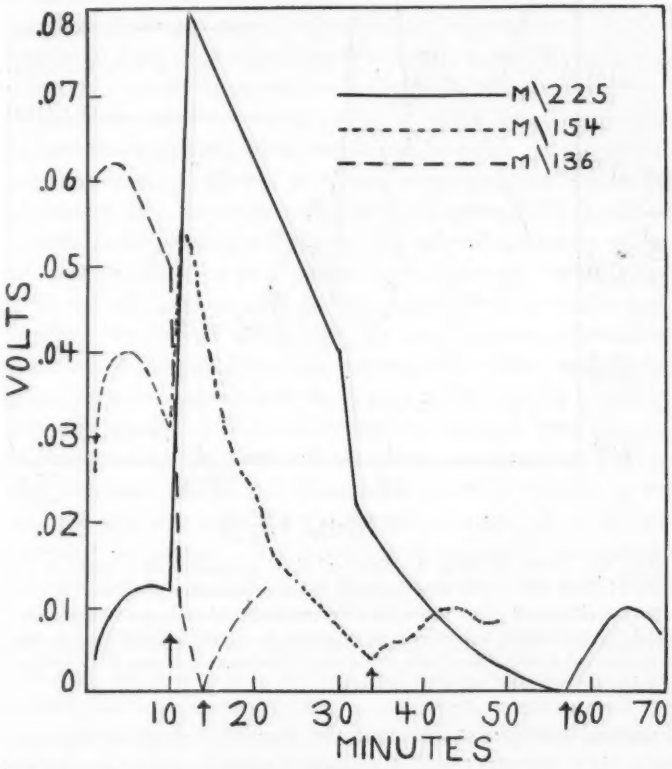

FIG. 10. Curve showing the relation of concentration of HCN to the P.D. of frog skin. Concentration of total cyanide = $M/225$, $M/154$ and $M/136$. Same solution on both sides of the membrane. The period between arrows indicates the time that the skin was exposed to cyanide, at other times the skin was exposed to borax buffer free from HCN. Abscissæ represents the time in minutes; ordinates the P.D. in volts. Different pieces of skin from the same frog were used.

recovered when removed from the cyanide. It is obvious from Fig. 10, that a weak solution of cyanide acted at first as a stimulant and this was followed by a delayed toxicity. As the concen-

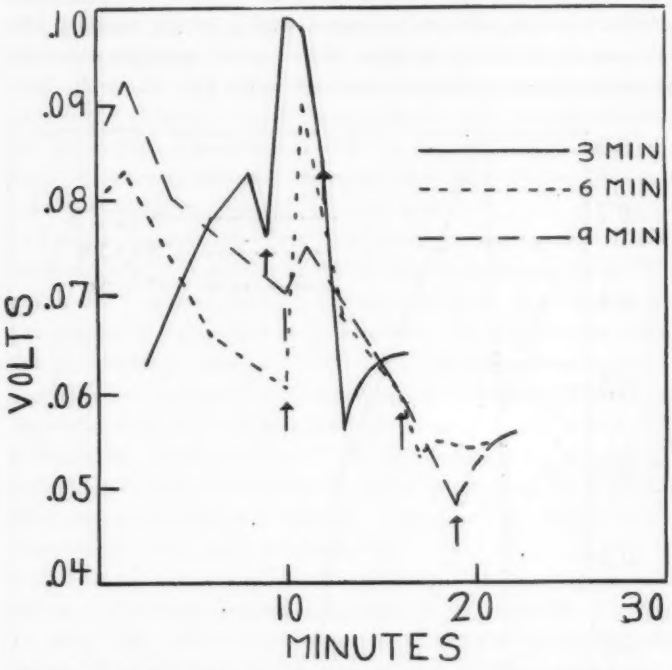

FIG. 11. Curve showing the effect of short exposures of cyanide to the P.D. of frog skin. The time between arrows represents the period of exposure to HCN, at other times the skin was exposed to borax buffer alone. Solid line represents a three minute exposure to cyanide; dotted line, 6. min. exposure and broken line 9 min. exposure. Abscissæ represents the exposure in minutes; ordinates, the P.D. in volts.

tration of cyanide was increased, the stimulation decreased and the toxic effect increased until a point was reached where stimulation no longer occurred and at this point the toxic action was pronounced. This initial stimulation of frog skin by cyanide was apparently overlooked by Lund (*7a*) who took readings every fifteen minutes. He did notice, however, a rapid rise followed by a rapid fall in the electrical resistance in Obelia during a period

of exposure to KCN. It is well known that many anesthetics in dilute solutions act as protoplasmic stimulants and in concentrated solutions their toxic action is established (Osterhout, 9).

It is interesting in this connection to determine whether the rapid fall in the potential difference immediately following stimulation by dilute cyanide is due to a natural recovery to normal or to the toxic action of the cyanide. Cells were removed from the borax buffer solution at the end of a ten minute exposure and placed in a hydrogen cyanide solution of a concentration of $M/160$. A series of three experiments was conducted; in the first series the cells were removed from the cyanide solution at the end of three minutes and placed in a borax buffer, at which time the stimulation had reached its maximum and started to drop. In the second series the skin was allowed to remain in the cyanide solution for six minutes, then removed and placed in the pure buffer, at which time the drop following stimulation had reached the same reading as when the skin was first placed in the cyanide. In the third series the skin was exposed to the cyanide for a period of nine minutes. At the end of that time, the drop following stimulation had reached a point below the original P.D. The results of a typical series of experiments are plotted in Fig. 11. It is evident from this figure that after the removal of the skin from the cyanide solution, in the three and six minute exposure, the drop in P.D. continued until it had fallen below the P.D. obtained at the time the skin was placed in the cyanide solution; the P.D. then increased to normal. The nine minute exposure showed no further drop in the P.D. after being removed from the cyanide solution but an immediate recovery occurred. It is apparent from the data given that the drop in potential difference immediately following the stimulation was due not to the toxic action of the cyanide but to a natural return to the original reading and the toxic action did not take place until after the drop had surpassed the point where the skin was stimulated.

Effect of the pH of Cyanide Solution on the Potential Difference.

It is apparent from the data given in the first part of this paper that little or no HCN penetrates living membranes except in the

form of undissociated molecules; thus it was deemed advisable to study the effect of dissociated and undissociated molecules of HCN on the potential difference of frog skin. Control experiments were conducted using borax-boric buffer free from cyanide at a pH of 6.8 and 8.5 to determine whether or not the hydrogen ion has any effect on the frog skin. Fig. 12 shows the results

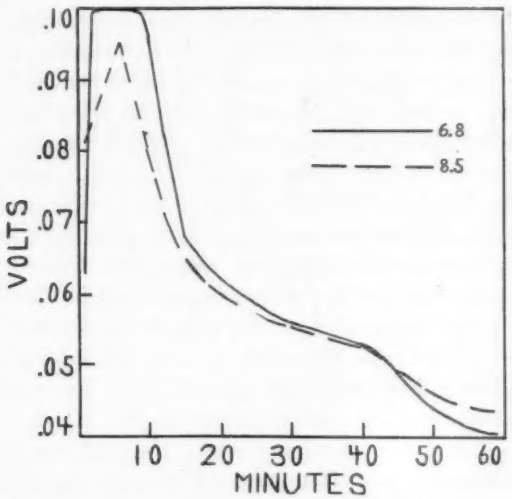

FIG. 12. Curve showing the effect of pH of the borax buffer on the potential difference of frog skin. pH valves 6.8 and 8.5. Abscissæ represents the exposure in minutes; ordinates, the P.D. in volts.

obtained. It is obvious that the two curves coincide very closely and that the hydrogen ion had no effect on the skin. It may be noted that the curves are strikingly similar to the control curves plotted in Fig. 9. The initial rise followed by a gradual decline was again manifested. Fig. 13 shows the results obtained by placing the frog skin "cells" in a solution of HCN in borax buffer at pH values of 6.8 and 8.5. The concentration of cyanide used was M/154. The temperature was constant at 22° C. ± 0.5°. The "cells" were placed for ten minutes in a borax buffer solution at pH values of 6.8 and 8.5 respectively; at the end of that time they were removed and put in a borax buffer containing

HCN at their respective pH values. The period of exposure to was greater at a pH of 8.5 than at 6.8. It is also apparent that the drop in the P.D. after a return to normal was somewhat more cyanide is represented on the curve by the time between the arrows. From Fig. 13, it is obvious that the initial stimulation pronounced under the acid conditions than under the alkaline.

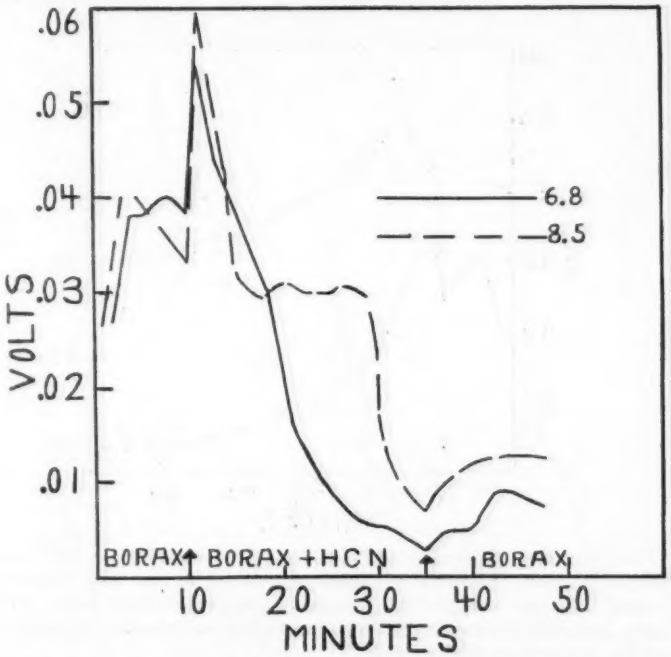

FIG. 13. Curve showing the effect of the pH of a solution of HCN in borax on the P.D. of frog skin. Period of exposure to cyanide represented as the time between arrows, at other times the skin was exposed to borax alone. pH values used were 6.8 and 8.5. Abscissæ represents the exposure in minutes and the ordinates the P.D. in volts.

There actually are fewer molecules of cyanide in a solution with a pH of 8.5 than with a pH of 6.8 (Fig. 1). So if the molecules were the toxic units, it would be expected that there would be less stimulation and more toxicity when the solution contains the greatest number of molecules than under the reverse conditions of

less molecules and more ions. Such a condition was found to exist, as is evident from Fig. 13 where more stimulation and less toxicity occurred under the alkaline conditions than in the acid solutions.

In Fig. 14 are plotted the results of a series of tests using a solution of HCN in Ringer's solution. The pH of Ringer's solution was about 8.2; by the addition of HCl the pH was changed to

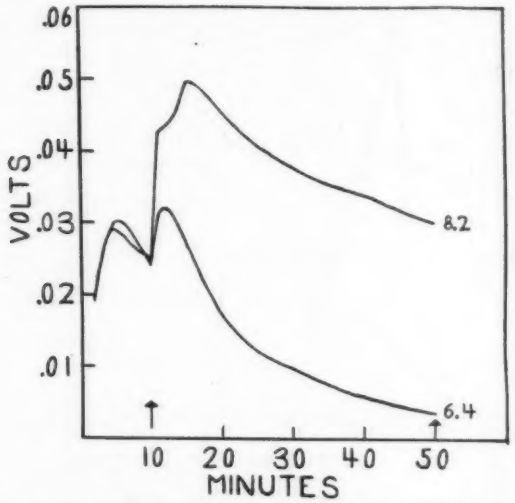

FIG. 14. Curve showing the effect of the pH of a solution of HCN in Ringer's solution. Exposure to cyanide represented by the time between arrows. First ten minutes skin exposed to Ringer's solution alone. pH values used were 6.4 and 8.2. Abscissæ represents the exposure in minutes and the ordinates the P.D. in volts.

6.4. The same concentration of cyanide was used in each series ($M/250$). The "cells" were placed in Ringer's solution for ten minutes, then removed; one series was placed in a solution of HCN in Ringer's solution at a pH of 6.4 and the other series at a pH of 8.2. Potentiometer readings were taken every minute for 45 minutes. Again it is evident from Fig. 14, that there is greater stimulation and less toxicity under alkaline than acid conditions. In either case the initial stimulation was not as pronounced as in the experiments when borax buffer solution was used. It is, how-

ever, sufficiently marked to be significant. Figs. 15 and 16 show the results obtained when the pH was alternated from 6.6 to 8.2 and vice versa. The same piece of skin was used under the same conditions of temperature and concentration of cyanide; the concentration of cyanide was $M/173$, made by adding liquid HCN to

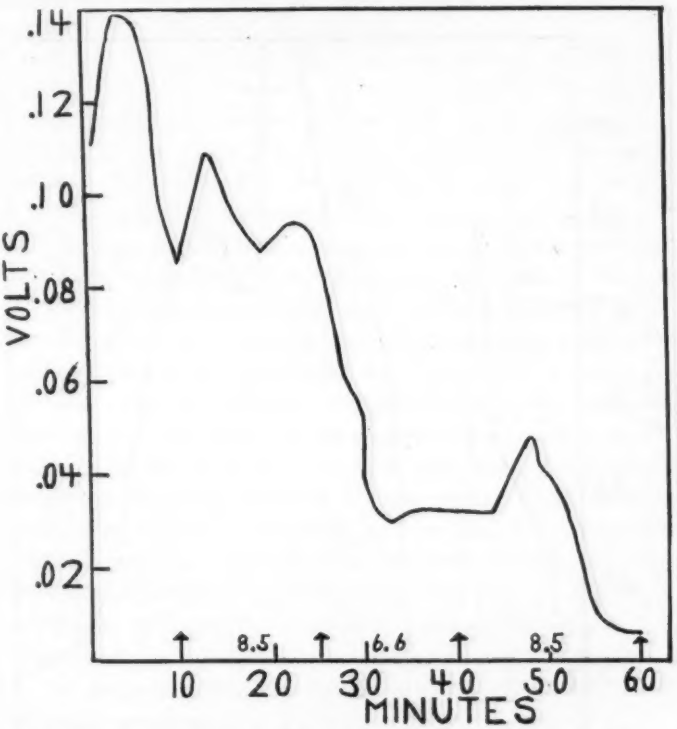

FIG. 15. Curve showing the effect of alternating the pH of a solution of HCN in borax on the same piece of frog skin. pH changed from 8.5 to 6.6 then back to 8.5. Period of exposure at various pH value indicated in curve. For the first ten minutes the skin was exposed in minutes and the ordinates, the P.D. in volts.

a borax buffer. The skin was placed in a borax solution for ten minutes, then removed and placed in the cyanide solution at a pH of 8.5 for 15 minutes (Fig. 15). The characteristic curve was obtained—an increase in P.D. followed by a gradual decrease.

When the period of 15 minutes had elapsed, the skin was removed and placed in a HCN solution of the same concentration and at a pH of 6.6. It will be noted from Fig. 15 that a very sudden drop occurred in the P.D. as soon as the skin was placed in the solution at a pH of 6.6. At the end of 15 minutes the skin was again placed in the original HCN solution at a pH of 8.2.

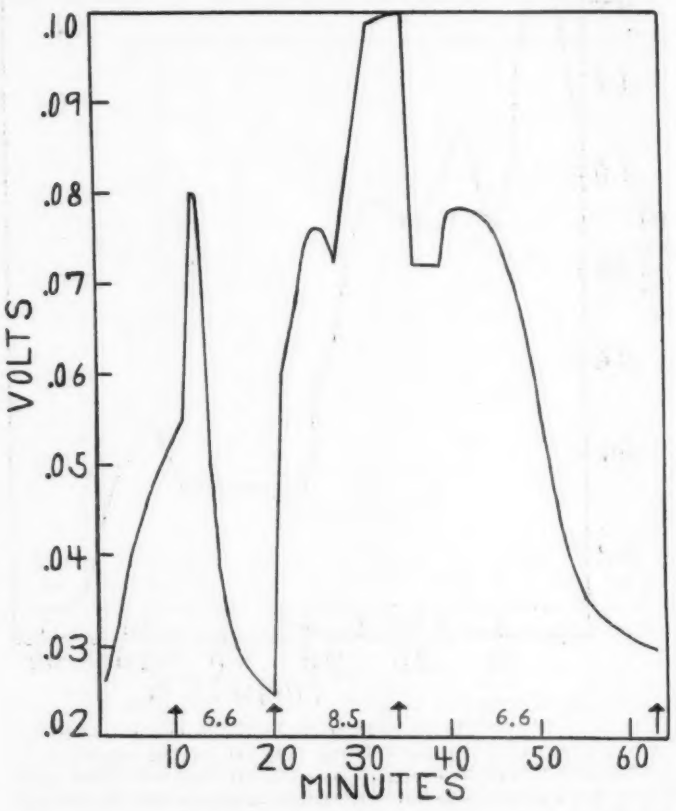

FIG. 16. Curve showing the effect of alternating the pH of a solution of HCN in borax on the same piece of frog skin. PH changed from 6.6 to 8.5 then back to 6.6. Period of exposure at various pH values indicated in curve. For the first ten minutes the skin was exposed to borax alone. Abscissæ represents time of exposure in minutes and the ordinates the P.D. in volts.

The P.D. gradually increased—suggesting a recovery—followed by a gradual decrease to the base line. The curve suggests, that at a pH of 6.6 the cyanide was more toxic than at a pH of 8.2 as was evident from the fact that the P.D. suddenly dropped when the skin was taken from a solution of a pH of 8.2 and placed in a pH of 6.6. When the skin was taken from a pH of 6.6 and placed in a solution of 8.5 a recovery occurred.

The conditions plotted in Fig. 16 are the reverse from those in Fig. 15. The skin after being placed in a borax buffer solution for ten minutes was removed and put in a cyanide solution of a pH of 6.6. The initial rise followed by a rapid drop was again obtained. When, however, the skin was removed from the pH of 6.6 and placed in a cyanide solution having a pH of 8.5, the P.D. rapidly increased until a point was reached above the initial stimulation suggesting a recovery and a stimulation. At the end of 15 minutes the skin was again placed in the original HCN solution at a pH of 6.6; a rapid fall occurred, suggesting a return to normal, followed by a rapid decline. As previously stated, 98 per cent. of the total cyanide is dissociated at a pH of 8.5 and thus the cyanide is present in the ionic condition. When the pH is 6.6 the cyanide is about 40 per cent. dissociated, therefore, the number of molecules and ions is about equal. From the data given, it appears that the cyanide is more toxic to the frog skin when the solution is acid than when alkaline. Thus, it seems that the molecule is actually more toxic than the ion. In dilute concentrations the physiological effect of hydrogen cyanide on frog skin is first a stimulation followed immediately by a rapid return to the original reading terminating in a toxic effect which will eventually prove fatal.

Summary.

The experiments indicate that little or no HCN penetrates frog skin "cells" except in the form of undissociated molecules. The total amount of intracellular cyanide is proportional to the concentration of undissociated molecules in the external solution. The internal pH value of the "cell" has no effect on the penetrations of HCN through frog skin.

From a study of the effect of hydrogen cyanide on the potential

difference of frog skin, it appears that dilute solutions of cyanide cause an initial stimulation followed by toxicity. As the concentration is increased, the stimulation is decreased and the toxicity is increased until a certain concentration of cyanide is reached where there is no stimulation but a marked toxic effect is evident.

The data also indicate that the molecule is more toxic than the ion.

BIBLIOGRAPHY.

1. **Bernard, Claude.**
'57 Lecons sur les effets des Substances toxiques.
2. **Allen, G. W.**
'19 Am. Jour. of Phy., Vol. 48, p. 93.
3. **Child, C. M.**
'19 Am. Jour. of Phy., Vol. 48, p. 372.
4. **Hyman, L. H.**
'19 Am. Jour. of Phy., Vol. 48, p. 345.
4*a*. **Hyman, L. H.**
'16 Am. Jour. of Phy., Vol. 40, p. 328.
4*b*. **Hyman, L. H.**
'19 Biol. Bull., Vol. 37, p. 404.
5. **Vernon, H. M.**
'06 Jour. of Physiology, Vol. 35, p. 53.
6. **Buchanan, J. W.**
'26 Jour. Exp. Zoöl., Vol. 44, p. 285.
7. **Lund, E. J.**
'21 Am. Jour. of Phy., Vol. 57, p. 336.
7*a*. **Lund, E. J.**
'26 Jour. Exp. Zoölogy, Vol. 44, p. 383.
8. **Heilbrunn, L. V.**
'20 Biol. Bull., Vol. 39, p. 307.
9. **Osterhout, W. J. V.**
'17 Bot. Gaz., 63, p. 77.
9*a*. **Osterhout, W. J. V.**
'13 Science, Vol. 37, p. 111.
9*b*. **Osterhout, W. J. V.**
'25 Jour. Gen. Phy., Vol. 8, p. 131.
9*c*. **Osterhout, W. J. V., and Dorcas, M. J.**
'25 Jour. Gen. Phy., Vol. 9, p. 255.
9*d*. **Osterhout, W. J. V.**
'22 Injury, Recovery and Death. J. B. Lippincott.
10. **Geppert.**
'89 Zeitschr. f. klin. Med., XV., p. 208.
11. **Dontas.**
'08 Arch. Exper. Path. and Pharm., LIX., p. 430
12. **Child, C. M.**
'15 Senescense and Rejuvenescence, Chicago.

13. **Lillie, R. S.**
'18 Am. Jour. of Phy., XLV., p. 406.
14. **Lullies, H.**
'15 Pflügers Arch., 207, p. 8–23.
15. **McClenden, J. F.**
'15 Am. Jour. of Phy., Vol. 38, p. 173.
16. **Wertheimer, E.**
'23 Pflügers Arch., 201, p. 438.
17. **Krehan, M.**
'14 Internat. Zeitschr. f. Physik and Chem. Bull., Vol. 1, p. 189.
18. **Jacobs, M. H.**
'22 Jour. Gen. Phy., 5, 181.
18*a*. **Jacobs, M. H.**
'20 Am. Jour. Phy., Vol. 41, p. 321.
19. **Finlay.**
'24 Physical Chem. for Students of Med. Longman, Green & Co.
20. **Michaelis und Rena.**
'13 Biochem. Zeitschr., Vol. 49, p. 232, and Vol. 67, p. 182.
21. **Brooks, M. M.**
'26 Am. Jour. of Phy., Vol. 76, p. 360.
21*a*. **Brooks, M. M.**
'26 Am. Jour. of Phy., Vol. 76, p. 116.
22. **Scarth, G. W.**
'24 Science, 60, p. 431.
23. **Collett, M. E.**
'19 Jour. Exp. Zoöl., Vol. 29, p. 443.
24. **Beerman, H.**
'24 Jour. Exp. Zoöl., Vol. 41, No. 1, p. 33.
25. **Bodine, J. H.**
'24 Jour. Gen. Phy., Vol. VII., p. 19.
26. **Feldman.**
'23 Biomathematics. Charles Griffin & Co., London.

SEX RATIO IN *GAMBUSIA*.[1]

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INTRODUCTION.

The unequal division of the sexes in adult *Gambusia* and other viviparous minnows is a subject discussed by many writers. The present author has had an opportunity, in connection with the study of the value of *Gambusia* as an agent for the control of mosquitoes, to make many observations and to sex numerous fish consisting of both adults and juveniles and the results from this work form the basis for the present paper.[2] The material upon which the present study was based was obtained chiefly in three localities, namely, Augusta, Ga., Beaufort, N. C., and Greenwood, Miss. Therefore, the data, according to the most recent classification (Geiser, 1923, and Hubbs, 1926), are based on two species of *Gambusia,* namely *holbrooki* from Augusta and Beaufort, and *affinis,* according to Geiser, or *patruelis* according to Hubbs, from Greenwood. The present writer is unable to discuss the merits of the further division between *affinis* and *patreulis,* recently recognized by Hubbs. However, the differences in the structure of the distal part of the anal fin of adult males from Augusta and Beaufort (Atlantic drainage) and those from Greenwood (Mississippi River drainage) are unmistakable and they are correctly described and figured by Geiser (1923). Furthermore, the present writer finds that when the rays in the dorsal fin are accurately and uniformly enumerated in both species, that is, when the last two rays that are more or less united, are counted either as one, or as two rays, a constant difference of one ray becomes evident.

[1] Published by permission of the U. S. Commissioner of Fisheries.

[2] The writer wishes to acknowledge the very valuable assistance rendered by Irving L. Towers, recently junior aquatic biologist, U. S. Bureau of Fisheries, who was associated with the author for several years in the *Gambusia*-mosquito studies and who personally sexed many adult fish and made numerous observations herein recorded.

When counting the last, partly united, rays as two in 81 specimens examined from the Atlantic drainage (Augusta, Ga., Beaufort, N. C., Orangeburg, S. C., and Key West, Fla.) the dorsal fin constantly had 8 rays. Similarly, in 78 specimens examined from the Mississippi River drainage (Greenwood, Miss., Memphis, Tenn., Mound, La., and Little Rock, Ark.) 7 dorsal rays invariably were present. The variation of two and three rays reported in published works, therefore, probably is due chiefly to a difference in enumeration. The difference in the number of dorsal rays, then, is helpful in separating the otherwise bothersome females, as well as all juveniles which have not yet developed the external sexual characters.[1]

Although the data, forming the basis for the present paper, are founded upon two species of *Gambusia,* they will not be delt with separately, as no differences between the species with respect to sex ratio, neither seasonal, for adults, nor for young fish were noticed. The specimens, collected from 1921 to 1926, were taken in a large variety of waters, that is, ponds of various sizes (with and without vegetation) borrow pits, swamps (both fresh and brackish), ditches and sluggish creeks. The specimens, in fact, were taken in nearly every environment in which *Gambusia* lives. The data, therefore, are representative of animals living under a large variety of conditions, and not of any particular environment.

Although many authors, as already stated, have discussed the sex ratio in adult Pœciliids, little work has been done on the sex ratio of immature fish. The only account of the sex ratio of young Pœciliids, of any importance, that has come to the writer's notice is the one by Geiser (1924). Even Doctor Geiser's data are rather limited, yet they show quite accurately the sex ratio of young *Gambusia,* as will be demonstrated by the extensive data that are offered in a subsequent section of this paper.

[1] It appears to be of interest to note here that a small collection (26 specimens) from Camilla, Ga., is at hand in which occur five females with only 7 rays in the dorsal fin, whereas all the others, including two males, have 8 rays. Geiser (1923) studied specimens from the same collection and, basing his identification on the minute structure of the intromittent organ of adult males, found them to represent the eastern form, *holbrooki,* although Camilla is in the Gulf drainage. It would now appear as if both species occur in this locality. Unfortunately no males with 7 dorsal rays are at hand with which to verify this identification.

Sex Ratio in Adult *Gambusia*.

The literature dealing with the sex ratio of adult Pœciliids, as previously indicated, is extensive and scattered and it will not be reviewed here. The reader, however, is referred to Dr. Samuel W. Geiser's admirable paper, "Sex-ratio and Spermatogenesis in the Top Minnow, *Gambusia holbrooki* Grd.," (1924) for a review of this literature and a concise discussion on the subject. It is sufficient to say here that the inequality of the sexes in adult fish is an unmistakable and a positive fact. As further proof, Table I., based on 103,150 fish, examined for sex, is offered. In explanation, it may be stated that the fish were collected either with a bobbinet seine or a dipnet made of the same material. Therefore, it was impossible for the smaller males to escape through the meshes of the nets.

In general fish having a total length of about 21 mm. (using "eye measure") and over were considered adult fish and are included in Table I. The modified anal fin, serving as a copulatory organ, of course, was used in separating the sexes. That is, when a fish possessed a modified anal fin it was classed as a male, otherwise as a female. Many males, as will be explained later, undoubtedly were classed as females, because of the late development of the copulatory organ in some individuals. Occasionally this organ is fully developed when the fish is 18 mm. long and generally if not fully formed it is at least sufficiently developed to admit of recognition when the fish is about 21 mm. long. However, many exceptions are found, as is shown in the section of this paper dealing with the sex ratio of immature fish. It is enough to say here, that the disparity of the sexes, that is, the minority of males, is not as great as shown in Table I. The number of males, classed as females, nevertheless, is entirely too small wholly to account for the large inequality in the sexes, and the fact that the males are greatly in the minority among adult fish unquestionably remains.

It was shown by Barney and Anson (1921) that a "seasonal male frequency" takes place. The data presented in Table I. bear out the information offered by these writers, namely that the males are comparatively much more numerous during the fall,

winter and spring than they are in midsummer. The data at hand are for the following months: June, July, August, September, October and December. Males were most numerous, as shown by these data, in June, when the ratio was 1 male to 2.54 females among 4,902 fish examined. In July the ratio, based on the examination of 17,941 fish, was 1 male to 5 females. In August the males were the fewest, as a ratio of only 1 male to 11.3 females obtained, as shown by the examination of 21,446 fish. In September the males began to increase, for a ratio of 1 male to 8.35 females was obtained from a total of 13,473 fish. In October the males increased still further, for 1 male to 2.75 females was found among 43,288 fish examined. A single day's collection, from Beaufort, N. C., taken chiefly in brackish swamps, for December is at hand, which consists of 2,100 fish. The sex ratio in this collection consists of 1 male to 3.6 females. The number examined obviously is too small and the environment too limited to be representative of the usual sex ratio prevailing at this season of the year. The average ratio of males to females for all fish (103,120) examined is 1 to 4.4.

The comparatively large seasonal difference in sex ratio in *Gambusia,* is very interesting. Barney and Anson (1921) offer the following explanation (pp. 64 to 66): "Further inquiry into the causes of the varying seasonal frequency of males and of the species, shows that in the summer there is a decrease in depth and area of all water systems studied. . . . This lowering of the water in the bayous and ponds eliminates all the shallow margins which *Gambusia* frequent and where they are largely immune from their enemies, and forces them into deeper water where the incline of the banks is steeper. This accordingly means much less natural protection than the minnows formerly had, since predaceous fish, now at a period of heightened metabolism and consequent rapid growth, are especially destructive and have a marked effect in lowering *Gambusia* frequency. Doubtless the *Gambusia* eaten by their predators at this time are nearly all females of large size, since the adult females, gravid at this season, are much larger, much slower in movement, and are more noticeable because of their black abdominal spots than the small, quickly moving, uniformly colored male. With the height of *Gambusia* frequency

in late August (1919) occurs properly the height of the young *Gambusia* frequency. The young frequency then diminishes quickly, apparently due to the scarcity of breeding males during the spring and summer, and the resulting lowered birth rate. The height of *Gambusia* frequency is immediately followed by a considerable drop in water stage (surface water index) and accompanied by an increase in the percentage of other species, many of which are predators. The *Gambusia* frequency decreases by loss of adult females, while the male frequency accordingly increases." In the light of the present studies, as well as the tables presented by Barney and Anson (1921) this explanation is entirely inadequate, and in the main contrary to what actually takes place. If, in the thinning out process, more females than males were eliminated, then, certainly, there should be an abundance of males in July and August. That is, at a time when few young of the current season have become sexually mature and when it is usually quite possible to distinguish between the young of the current season and those of the previous year. Barney and Anson's table, as well as Table I. (presented herewith), however, show that the reverse unmistakably is true. Furthermore, it may be remarked here that the error in sexing, by means of external characters, very probably is smaller during July and August than at any other time. That is, fish of the previous season have become sexually mature and comparatively few young of the current season have reached a sufficiently large size to be considered adults. Therefore, comparatively few males, at this time, are classed as females.

How may the seasonal variation in sex ratio of *Gambusia* then be explained? It is shown in a subsequent section of this paper that males and females are equally proportioned among young *Gambusia,* and it already has been stated that a very large decrease in the proportionate number of males present takes place shortly before (July and August) the main body of young fish of the current season become sexually mature. A greater thinning out of adult males than of adult females, therefore, must have taken place. A discussion of the evidently much more rapid decrease among the males than among the females, when few young fish are maturing to take their place, is reserved for a later section. It appears to be sufficient to state here that the proportionately

greater abundance of males which begin to appear in September are the result of young fish that are becoming sexually mature, for among these fish the males are quite as numerous as the females. After the following May or June nearly all of the fish, born the previous year, have become sexually mature and the young of the current season are still nearly all immature fish. Then the very rapid decline in the male population, already mentioned, takes place.

Further study has convinced the writer that the main reproductive season of *Gambusia* is much more limited than stated by him (1917 and 1921). In catching and transporting large numbers of fish while engaged in the study of *Gambusia* in its relation to mosquito control, it became evident that, although some gravid females are seen in early spring and during the fall,[1] the main reproductive season lasts only about three months, namely June, July and August. A very sudden decrease in the number of gravid females was noticed at Augusta, Ga. (where special attention was given to this matter for several years in succession) during the early days of September and in that locality, at least, the reproductive season may be said to end by Labor Day. The end of the spawning period evidently is not greatly influenced by temperature, as has been the contention of various writers, for the month of September at Augusta generally is one of the warmest of the summer. Furthermore, the writer has for two winters (1925–26 and 1926–27) kept two lots of adult *Gambusia,* containing a fair percentage of males, in the terrapin nursery house at the U. S. Fisheries Biological Station, Beaufort, N. C., which is kept at summer heat. Yet reproduction was not induced during the winter months. It was noticed, however, that gravid females appeared somewhat earlier in the spring in the lots in the terrapin house than among fish that wintered out of doors. The influence of temperature on reproduction in *Gambusia,* therefore, does not appear to be very pronounced.

The scarcity of gravid females in "midsummer" was noticed by Barney and Anson (1921). These authors thought this to be due to the scarcity of males, stating that such a "condition would

[1] In the extreme southern parts of the United States, as at Key West, some gravid females are present at all seasons of the year.

not normally occur in midsummer with a proper relative number of males present." These authors probably overlooked the fact that copulation is not necessary between broods in *Gambusia*, nor in certain other Pœciliids, as it has been shown by Zolotnisky (1901), Philippi (1908) and Hildebrand (1917) that several broods of young may be produced by females after they are separated from males. For example, Hildebrand (1917) segregated some female *Gambusia* early in the spring, providing them with individual aquaria. One female, at least, produced five broods of young that summer, without further copulation. If copulation is not necessary during the summer, as appears to be the case, then certainly the scarcity of males cannot account for the cessation of reproduction during midsummer. Furthermore, the writer has held both sexes of *Gambusia* together in aquaria for a number of years and he has not noticed that the reproductive period is lengthened thereby. It appears to be logical, therefore, to conclude that the great reduction in the proportionate number of gravid females at the end of August is not due to the scarcity of adult males. On the contrary, it is the opinion of the writer that the animals by that time have expended all of the energy on reproduction they can afford and breeding ends, regardless of weather conditions.

TABLE I.

THE SEX IN ADULT *Gambusia*.

Month.	Males.	Females.	Ratio.
June	1,385	3,517	1 : 2.54
July	2,960	14,981	1 : 5.06
August	1,742	19,704	1 : 11.31
September	1,440	12,033	1 : 8.36
October	11,614	31,674	1 : 2.75
December	455	1,645	1 : 3.6
Total	19,596	83,554	1 : 4.4

SEX RATIO IN IMMATURE *Gambusia*.

It already has been pointed out that the sex ratio in young fish has received comparatively little attention. This is rather surprising in view of the many accounts that have been published dealing with the sex ratio of adult Pœciliids. Perhaps this interesting problem did not receive more attention for the want of

proper material and because of the difficulty involved in determining the sexes in young fish. Sexing immature fish, however, was found easier than the writer had anticipated, and a brief description is offered of the technique pursued.

Sexually mature fish at first were dissected with the view of learning definitely the exact position of the gonads and to study the general appearance of these organs in fish in which the sex was definitely known. It was found that with scissors the tail and a part of the back of the fish might be removed by a single clip and by making this cut through the base of the anal and sloping it forward at an angle of about 45 degrees the viscera, after removing the peritoneum, was exposed while remaining intact. The sexual organs, lying dorsally of the visceral mass, may then be examined in position or removed for examination. It will be noticed at once that the ovary has a black membranous covering, whereas, the testes in preserved specimens are pale in color. The testes, although lying very close together and described as " fused " by Geiser (1924), nevertheless, show distinctly a median depression and on this line they may be separated with a sharp needle into two nearly equal parts. The ovary, on the other hand, is definitely fused, it has no median line of depression and cannot be separated into equal parts without causing rough, unequal breaks. The more prominent projection into the abdominal cavity of the interhæmal spines in the male is another difference that is usable. These characters can be used in sexing immature fish from 15 mm. and upward in length, as they are evident under a binocular microscope.

In sexing fish that are less than 15 mm. long it is necessary to be more careful in removing the minute gonads and for differentiation they must be placed on a slide. As it is necessary to use transmitted light, the contents have to be spread. This may be accomplished by placing a cover glass over the glands and tapping it lightly. It is desirable at first to mount and study the gonads of somewhat larger fish, that is, of fish large enough to admit of sex determination by the method described in the preceding paragraph. This is desirable, because confusion may arise from the fact that the sperm cysts might be regarded as eggs. It will be noticed, however, under proper magnification that the cysts are more

opaque and granular in appearance than the ova and they do not have a central "nucleus" which is quite characteristic of the eggs. It is possible to sex *Gambusia* at birth, that is when only about 8 mm. long by this method. Consequently, no tedious processes of embedding and sectioning of gonads need be involved. After a little practice one becomes quite proficient in removing the organs, as well as certain of the diagnosis.

It of course is understood that the specimens, especially the very small ones, must be well preserved or sexing by the process described becomes difficult and unreliable. The best results were obtained by the use of 80 per cent. alcohol to which 40 per cent. formaldehyde was added in the proportion of about 30 cc. to 400 cc. of the alcohol. The strength of the preservative of course may be considerably varied according to the temperature. During cool weather 80 per cent. alcohol alone preserves specimens quite as well as the mixture of the two, in the proportions given, during hot weather.

The rapidity with which immature fish could be sexed, in the manner described, made it possible to examine a comparatively large number of fish, as shown in Table II. Few males have become sexually mature at a length of 20 mm. and no appreciable change in the ratio of males to females has taken place in fish of this size. This is brought out in Table II., in which fish, 20 mm. and under in length, are divided into two groups, namely those of 15.5 to 20 mm. being placed in one group and those of 15 mm. and under in another. It will be seen that in each group the males and females are very nearly equally numerous. This seems also to show that no thinning out of males takes place before they have become sexually mature.

The data for the next group consisting of larger fish (20.5 to 25 mm.) listed in Table II. are not representative of the sex ratio in unselected fish of that size. It is understood that many fish 20.5 to 25 mm. in length are sexually fully mature, the males being recognizable by the modified anal fin and the females, if not actually gravid, nevertheless, frequently have a small elongate blackish spot or line in the position where the prominent black spot is situated when the abdominal walls are distended with eggs and young. Such fish are not included in this group, as it is based

only on specimens that had developed no external sex characters by means of which they could be definitely recognized. These data show that no hard and fast line, with respect to size, can be drawn between adult and immature fish, as the external sex characters are developed at a much larger size in some individuals than in others. In this group of large immature fish, based on 1,660 specimens, the males, however, are considerably in the minority, as the ratio is 1 male to 1.48 female.

A few immature fish of even a larger size than the group discussed in the preceding paragraph were found. For example, among 285 fish, ranging from 25.5 to 30 mm. in length which could not be sexed definitely from external characters, the ratio was 1 male to 6.12 females. In this connection it may be noted, however, that not a single immature male exceeding a length of 28 mm. was found.

The variation in size and age at which the anal fin in the male becomes differentiated, that is, when it is developed into an intromittent organ or "gonopod" (as designated by Geiser (1924)) already has been pointed out by Hildebrand (1917) and Mast in Barney and Anson (1921). The variation in size at which this organ may develop appears to be even greater than these writers supposed. It is evident from these data also that a considerable number of males are included among the females when all fish of about 21 mm. and over in total length are considered "adult," as the present writer did in sexing fish by the use of external characters, or as Barney and Anson (1921) did when they classed all fish of 15 mm. and over in length, to the base of the caudal, as adults. A considerable number of males, therefore, were classed as females in Table I. However, as stated elsewhere, the error in sexing is not large enough to cause the great minority of males that is shown in Table I. The greatest error in sexing, that is, the largest number of males classed as females, undoubtedly occurred during the months when the males were increasing most rapidly in proportionate numbers, as for example in September and October, for it is then that the largest number of young are maturing and many come within the "doubtful" size group. (The principal error is among fish 21 to 23 mm. in length.) Similarly, the error in sexing must be smallest when the fewest young

fish are maturing, as in July and August when the greatest disparity of the sexes occurs. The uncertainty of the sex determination from an external examination of some of the fish, classed as females in Table I., therefore, accounts for a part of the difference in sex ratio shown but probably only for a small part of it.

It is believed that ample evidence has been produced by the data in Table II. to show that a *one to one* sex ratio exists among young *Gambusia*. It would appear also from these data that this one to one ratio obtains at least until the fish become sexually mature. It is evident from Table I., however, that a "thinning out" process takes place among adult males which is very much in excess of the decrease among the adult females. A discussion of this interesting phenomenon is offered in the following section.

TABLE II.

THE SEX IN YOUNG *Gambusia*.

Size of Fish.	No. of Fish Sexed.		Remarks.
	Males.	Females.	
15 mm. and under..	239	232	
15.5 to 20 mm.....	878	853	
20 mm. and under..	181	210	This lot was not kept separate more definitely as to size in the original records.
Total.....	1,298	1,295	Very few fish of 20 mm. and under are sexually mature. These data, therefore, show the sex ratio among young *Gambusia*.
LARGER IMMATURE *Gambusia*.			
20.5 to 25 mm.....	672	988	Based on fish that were not yet sexually mature, although fish in these size groups usually have developed external sex characters.
25.5 to 30 mm.....	40	245	
Total.....	712	1,233	

A DIFFERENTIAL DEATH RATE OF THE SEXES IN *Gambusia*.

This subject is discussed at some length by Geiser (1924) who says, "The males are much less resistent to harmful environmental factors than females, and hence have a lower survival value." This author then continues to say that he has found

males killed much more readily in "high temperatures, high H-ion concentration, oxygen deficiency and concentrations of KCN." He also found a much higher death rate among males than females in *Gambusia* held in containers for shipment, as well as when confined in aquaria. Further along in the same discussion this author writes, "The males are smaller and hence are more liable to be devoured by small predaceous fish than the much larger female. Gravid female *Gambusia* in aquaria, also, attack and frequently kill the males." The present writer is in entire accord with Doctor Geiser's views and the data presented herein appear to lend support to his contentions.

The present writer in connection with his work dealing with fishes in relation to mosquito control has had occasion to make shipments of hundreds of thousand of *Gambusia*. It was noticed from time to time, that among the dead the males usually appeared to be proportionately much more numerous than the females. It, of course, was impracticable to sex fish that were being transferred from one place to another for breeding purposes, hence the percentage of deaths of each sex cannot be given for such shipments. The writer is certain, however, that at least usually the death rate among the males was higher than among the females.

In a large series of experiments performed for the purpose of determining the best method of catching and handling *Gambusia*, the adult fish were sexed and for these the results follow. The effects of high temperatures, also the effects of crowding the fish, as well as the results to be derived from the use of varying amounts of water in containers holding fish, were tested. Furthermore, an effort was made to determine, by the number of fish that survived after several days of confinement under identical conditions whether catching by seine or by dipnet was preferable.[1] The results with respect to the death rate for each set of experiments will not be given separately, as it was higher for males than for females for every condition tested. In some individual lots proportionately fewer deaths occurred among the males than among the females. Generally the reverse, however, was true, as already stated and as shown by the data that follow.

[1] The practical results derived from the experiments referred to were reported by Hildebrand (1925).

A total of 41,073 adult fish, consisting of 7,337 males and 33,736 females, were used in the experiments described in the preceding paragraph. The loss among the males was 29¾ per cent. and among the females it was 22⅓ per cent. This mortality, as a whole, was very high, that is, much higher than usually occurs in *Gambusia* that are transported from one place to another, because many of the fish, for the sake of experimentation, were held under very adverse conditions. For example, in working with the effects of high temperatures the containers were set in the sun for the purpose of determining at how high a temperature the fish could survive. The males and females, however, were subjected to the same tests and, therefore, the data should be nearly as representative of one sex as of the other. Two discrepancies, nevertheless, may be mentioned. First, in some lots all the males died before the experiments were concluded. In such cases the mortality of the females (for additional ones usually died) is greater than it would have been, if the experiments had been terminated when the last male had died. Second, it is probable that some immature males that exceeded a length of 20 mm. died and, if so, they were classed as females. Therefore, the percentage of deaths given for the females is subject to a small error, as it is somewhat higher than it should be for direct comparison with the percentage of deaths among the males. It may be concluded, then, that the differential death rate among the fish used in the experiments was greater than indicated by the data.

The data show that under artificial conditions the death rate almost constantly was higher among males than among females, and it seems probable, that the males are generally less resistant to harmful environmental factors and this probably is true in nature as Geiser (1924) has contended, as well as under artificial conditions.

The present writer has no other pertinent data to offer bearing upon the cause or causes for the greater decrease in males than females, occurring annually. Geiser (1924) argues that the smaller males are more liable to be devoured by small predaceous fish than the much larger females and he also points out that the female, herself, is an enemy of the male. Barney and Anson (1921), on the other hand, argue (see quotation, p. 393) that the

larger and more conspicuous female is the one suffering most from predatory fish. The writer believes that the former view may be correct but that in addition to fish, other natural enemies, such as water snakes, birds and insects should be considered as they all take part in destroying *Gambusia*.

Conclusion.

It has been shown quite conclusively that in immature *Gambusia* the sexes are evenly represented, as the 2,593 fish (all 20 mm. and under in length) that were examined, consisted of 1,298 males and 1,295 females. It was shown also, that a large "seasonal" variation in the abundance of males among adult *Gambusia* takes place, the data presented showing that the fluctuation in sex ratio may vary from 1 male to 2.5 females in June to 1 male to 11.3 females in August. Some evidence was produced tending to show that this great "thinning out" of males may be due to their lack of resistance to adverse environmental conditions, and the possibility that they are more extensively preyed upon by natural enemies, also, was advanced. What the greater mortality of the males may be due to, however, very largely, remains for further investigation.

Although male *Gambusia* are constantly fewer in number than females, it seems certain, nevertheless, that enough males for breeding purposes always are present. It has been shown that copulation between broods is not necessary, as females separated from males early in the spring produced young throughout the breeding season. In the spring when apparently the majority of the females are fertilized for the duration of the breeding season, the males are proportionately much more numerous. The greatest scarcity of males occurs in midsummer (July and August) when the females born the previous season have nearly all matured and quite certainly have been fertilized. At that time few males are needed. It, at least, is unquestioned that when conditions are favorable, *Gambusia* are quite capable of perpetuating their kind. Certainly few fish multiply more rapidly or become more numerous. The thinning out of males, therefore, appears to be nature's process of eliminating "surplus" animals.

BIBLIOGRAPHY.

Barney, R. L., and B. J. Anson.

'21 Seasonal Abundance of the Mosquito Destroying Top-minnow, *Gambusia affinis*, Especially in Relation to Male Frequency. Ecology, Vol. II., 1921, pp. 53–69. Brooklyn.

Geiser, Samuel W.

'23 Notes Relative to the Species of *Gambusia* in the United States. The American Midland Naturalist, Vol. VIII., Nos. 8–9, 1923, pp. 175–188, 18 figs.

'24 Sex-ratios and Spermatogenesis in the Top-minnow, *Gambusia holbrooki* Grd. BIOLOGICAL BULL., Vol. XLVII., No. 3, 1924, pp. 175–212, 3 pls. Lancaster, Pa.

Hildebrand, Samuel F.

'17 Notes on the Life Histories of the Minnows *Gambusia affinis*, and *Cyprinodon variegatus*. Appendix VI., Report of the U. S. Commissioner of Fisheries for 1917 (1918), 15 pp., 4 figs. Washington.

'21 Top Minnows in Relation to Malaria Control, with Notes on their Habits and Distribution. Public Health Bulletin No. 114, U. S. Public Health Service, 1921, 34 pp., 32 figs. Washington.

'25 A Study of the Top Minnow, *Gambusia holbrooki*, in its Relation to Mosquito Control. Public Health Bulletin No. 153, U. S. Public Health Service, 1925, 136 pp., 15 figs. Washington.

Hubbs, Carl L.

'26 Studies of the Fishes of the Order Cyprinodontes VI. Miscellaneous Publications, Museum of Zoölogy, University of Michigan, No. 16, 1926, 86 pp., 4 pls. Ann Arbor.